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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR

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STUDIES IN THE METABOLISM OF ACTINOMYCETES

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PART I

I. INTRODUCTORY

The Actinomycetes form an extensive and widely distributed group of microorganisms. Like the bacteria and the molds, they occur in nature both as saprophytes and as parasites of plants and animals. While the other two groups of microorganisms have received much attention, this group has been studied very little either morphologically or physiologically, so that it has not even been decided as yet whether its members should be classified with the bacteria, the fungi, or placed in a class by themselves. Although certain *Actinomyces* species have been known for a long time, it is only within very recent years that a thorough study has been made of the structural and physiological characters of the groups, suggestions presented as to the best methods of classification and attempts made to interpret their probable part in nature.

A complete historical review of the pathogenic forms can be found in the work of Musgrave, Clegg and Polk (1908), while the occurrence and activities of the saprophytes have been reviewed by Krainsky (1914), Waksman and Curtis (1916, 1918), and others.

The growth of the Actinomycetes on organic media is not characteristic and this was the reason why the older workers, limiting themselves only to cultural studies and superficial observations, reported very few species of Actinomycetes to be present in nature. The work of more recent investigators in which differentiation is based on the size and shape of spores

and colonies and pigment production, as by Krainsky (1914) or on the liquefaction of gelatin, size of spores and spiral production, as by Waksman and Curtis (1916), marks a step in advance from that of Beijerinck, Sanfelice, Rossi-Doria, Münter and others, who based their differentiation only on pigment production and cultural characters upon media not standard in composition.

The use of inorganic media for the study of this group of organisms marks another important stage in the proper differentiation and classification of these organisms. Several excellent media of standard composition have been suggested and used very successfully: calcium malate agar[1] and glucose agar used by Krainsky (1914); Czapek's agar by Waksman and Curtis (1916) and malate-glycerin agar by Conn (1917). These media are well adapted for the purpose of bringing out the characteristic macroscopic features of the organisms and also for morphological studies. But one should not limit himself merely to the study of morphological and cultural characters. The morphologist always comes first in the study of a new organism, but the physiologist soon follows and the facts brought out by the latter are of as great and often greater importance in supplying information concerning the nature of the organisms than the facts brought out by the former.

The Actinomycetes, like other living things, exhibit phenomena of variability often neglected by the students of microscopic forms of life. The species described are not absolutely fixed, either physiologically or morphologically. A certain physiological feature will depend not only on the species and the

[1] The composition of these media is as follows:

Ca-Malate agar: Water 1000 cc, agar 15 grams, Ca-malate 10 grams 1 per cent, NH_4Cl 0.5 gram per cent, K_2HPO_4 0.5 gram per cent.

Glucose agar: Water 1000 cc. agar 15.0 grams, glucose 10.0 grams, K_2HPO_4 0.5 gram, NH_4Cl,KNO_3, asparagin or peptone 0.5 gram per cent.

Czapek's agar: Distilled water 1000 cc., K_2HPO_4 1.0 gram, $MgSO_4$ 0.5 gram, KCl 0.5 gram. $FeSO_4$ 0.01 gram, $NaNO_3$ 2.0 gram, sucrose 30.0 gram, agar 15.0 gram.

The substitution of glucose or glycerin for sucrose makes this medium more favorable for the growth of these organisms.

Malate-Glycerin agar: Calcium Malate agar + 1 per cent glycerin.

medium, but on other environmental conditions and on the nature of the mother culture. When many strains are accumulated we find that one organism will approach another organism and often a culture is obtained which can hardly be differentiated from either of the two. The differences in physiological activities are often not qualitative but quantitative. The dominant idea in the following investigations has been not merely the accumulation of more data for the differentiation of the species, but the study of the metabolism of these species so as to find their proper place in nature, both as individuals and as groups consisting of allied species.

The uses of the terms *Actinomyces*, *Streptothrix* and *Nocardia*, have been reviewed by Waksman and Curtis (1916), Krainsky (1914) and Conn (1917); the term *Actinomyces* will be used for reasons given in the above mentioned papers.

A preliminary description of the organisms isolated from the soil by Waksman and Curtis has appeared elsewhere (1916) and a full description will be published at a later date.

The following organisms were used in these investigations:

A. violaceus-ruber W. & C.

A. violaceus-acesari W. & C.

A. scabies (Thaxter) Güssow (Syn. *Oospora scabies* Thaxter, *A. chromogenus* Gasperini?) isolated from potato scab.

A. exfoliatus W. & C.

A. diastaticus (Krainsky?) W. & C.

A. albus (Krainsky?) W. & C.

A. reticuli W. & C.

A. citreus (Krainsky?) W. & C.

A. griseus (Krainsky?) W. & C.

A. verne W. & C.

A. alboflavus W. & C.

A. albosporeus (Krainsky?) W. & C.

A. bobili W. & C.

A. californicus W. & C.

A. lipmanii W. & C.

A. rutgersensis W. & C.

A. aureus W. & C.

A. halstedii W. & C.
A. fradii W. & C.
A. roseus (Krainsky?) W. & C.
A. lavendulae W. & C.
A. poolensis Taubenhaus.
A. pheochromogenus Conn.

96, 120, 128, 145, 154, 161, 168, 190, 198, 202, 205, 206, 214, 215, cultures isolated from different soils by Waksman and Curtis.

For the following four cultures the writer is indebted to Dr. K. F. Meyer of the Hooper Foundation, San Francisco.

A. madurae: Received from A. M. N. H. by Parke, Davis Company (no. 05), received by them in May 1902, from Král.

A. hominis: Isolated by Foulerton from abscess of palm of hand in 1911.

A. bovis: Received from A. M. N. H. no. 49, received by them from Parke, Davis and Company (no. 04).

A. asteroides (*A. eppingeri*): Received from Pasteur Institute, 1914.

II. THE ACTION OF ACTINOMYCETES UPON MILK

Historical

The importance of milk as a culture medium for the study of bacteria has long been recognized by workers in bacteriology; although milk is a very complex medium, it is more or less standard in composition and the reactions produced upon it by microorganisms are so characteristic, that it has found general acceptance.

The growth of an organism upon milk should be studied from several angles: the action upon the proteins and sugars of the milk, and the production of different enzymes must all be considered. Nitrogen is present in fresh skim milk as casein, about 3 per cent, albumin, about 0.5 per cent, and other nitrogenous compounds, about 0.05 per cent. The casein is in the form of calcium caseinate, which is acid to phenolphthalein and neutral to litmus. The calcium present in the milk is just sufficient to keep the casein in solution; when a large excess of

calcium salt is added to the milk, the casein is precipitated; upon addition of acid to milk, the calcium is converted into the corresponding salt and the free casein is precipitated, producing the well known phenomenon of coagulation. The coagulation of the milk may be due to two different causes: it may result from the action of an acid upon the milk, as just pointed out, or the curdling may be due to enzyme action, as in the case of rennin (chymosin, lab), which transforms casein into paracasein and produces coagulation, in the presence of calcium salts.

Milk contains as high as 6 per cent of sugar, mostly lactose, but with small quantities of glucose also. A permanent acid reaction of the milk is induced by the organisms that ferment lactose, but if an organism can only utilize glucose the reaction is usually at first acid, later changing to alkaline due to protein decomposition or the production of carbonates. The accumulation of acid due to the fermentation of lactose may be sufficient to cause an acid coagulation of the casein, and this is very often the type of coagulation which milk undergoes as a result of the action of bacteria. Savage (1904) demonstrated that curdling of milk by *B. coli* is due, in almost all cases, to the formation of acid, presumably lactic acid by the bacteria. Curdling of the milk produced by the action of an enzyme can be easily distinguished from that produced by an acid: when the acid is neutralized, the latter type of curd will redissolve, but not the former: O'Heir (1906) has shown that an acid coagulum in milk is insoluble in weaker alkalies, but dissolves readily in stronger ones such as NaOH. Savage (1904) demonstrated further that rennin will not coagulate boiled milk, or only with great difficulty, this being due to the precipitation of soluble salts of calcium in the boiling. When freshly separated and pasteurized milk is used, it is possible that the bacteria left in the milk may ferment the lactose and that the lactic acid thus produced may affect favorably the action of rennin.

Besides these primary reactions induced by the growth of microorganisms in milk, secondary reactions have to be taken into consideration also: such as the peptonization of the coagulum. This is due to the proteolytic activities of the organism, which

splits the paracasein by means of an enzyme into proteoses, peptones, polypeptides, amino acids, and ammonia. The milk may also clear up, without any previous coagulation. Van Slyke and Bosworth (1916) have shown that in the souring of milk an increase of the nitrogen of the whey takes place, and that this increase is due to the albumin. Kendall and his associates (1914) studied the growth of a number of bacteria on milk; many different transformations were produced in the milk as a result of the metabolism of particular organisms. *B. coli* produced a marked lactic acid fermentation with acid coagulation of the casein; *B. proteus* did not ferment lactose but attacked the proteins and at the end of the third day the milk was coagulated and later peptonized; *B. subtilis* and *B. mesentericus* acted in a similar manner, the first producing an alkaline and the second an acid reaction, and so on. These different reactions upon the milk can be easily explained by the differences in the metabolism of these organisms.

It is assumed that the enzyme produced by the microorganisms that clot milk is of a nature similar to the animal rennin, lab, or chymosin. Duclaux (1907) elaimed that bacteria act upon milk in three different ways: first, by producing a rennet-like enzyme which coagulates the milk, without making it more available for their nutrition; secondly, by producing a casease, an enzyme which digests the clot; this enzyme acts also upon the albuminoids of the milk but more actively upon the casein; thirdly, the microorganisms may produce a decomposition of the milk by a process of nutrition; if the casease is in excess over the rennet, the milk in this case will be decolorized without any previous coagulation.

Babcock and associates (1899), differentiated between the enzymes of animal origin and those produced by bacteria. He stated that the products formed by the galactase normally present in the milk resemble those produced by the liquefying or peptonizing bacteria rather than those formed by the enzymes of animal origin; trypsin, pancreatin and pepsin produce no ammonia, while galactase and the proteolytic enzymes from bacterial cultures do produce ammonia.

The chemistry of the rennet action is still a disputed point. When coagulation takes place, the bulk of the casein is precipitated as paracasein, which is very similar to casein itself; whether the decomposition of casein occurs is still uncertain. Some investigators, such as Pawlow and Parastschuk (1904), Sawjalow (1905), Sawitch (1908), Gewin (1907), and others, believe that the action of pepsin and rennet result from the same enzyme, the clotting of milk being regarded as the commencement of peptic digestion; others, such as Nencki and Sieber (1901), Pekelharing (1902), Taylor (1909), and particularly Hammarsten (1908) are of the opinion that the two enzymes are not identical. Hammarsten (1915) further states that both enzymes act proteolytically, but under different conditions, rennet acting at a lower acid concentration than pepsin, and also that the optimum temperature for the two enzymes is different. The casein molecule is split up into a larger molecule (Käse) and a smaller (Molkeeiweiss). The large molecule is rendered insoluble by the presence of soluble calcium salts and forms the clot. Schryver (1913) and Mellanby (1913) consider the rennet clot as probably a combination of enzyme and protein. Bosworth (1913) and Van Slyke and Bosworth (1916) believe that the ferment breaks up the casein molecule into two molecules of paracasein, each half the size of the original molecule; and that small quantities of $CaCl_2$ render the paracasein insoluble. Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein; the action attributed to rennin may be produced by any proteolytic enzyme.

Without going into further detail in regard to the chemical transformations of the milk constituents and the rôle of microorganisms in these transformations, a few words should be said here concerning the use of milk for the study of the Actinomycetes by the earlier investigators. Lehmann and Neumann (1912) reported that *A. bovis* Harz was found to produce no change in milk in eight days; *A. farcinicus* Gasperini dissolved the casein without coagulation, the reaction becoming alkaline; *A. chromogenus* Gasperini clarified the milk giving an alkaline reaction. Petruschky (1912) reported that *A. madurae* Vincent

coagulated the milk and later redissolved it, while Besson (1912) stated that *A. madurae* grew in milk, but that no coagulation took place. Goadby (1903) found that *A. buccalis* isolated from the mouth produced no change for the first two days in milk at 37.5°C.; later the milk was precipitated and then cleared, the reaction becoming slightly alkaline. Lutman and Cunningham (1914) found that *A. chromogenus* (*A. scabies*) digested the milk slowly without coagulum, the milk becoming alkaline. These few observations do not throw much light upon the action of the Actinomycetes upon milk, the only point that seems definite being the tendency for the reaction of the milk to become more alkaline as a result of the action of these organisms. All the Actinomycetes, according to Krainsky (1914), attack casein readily and split it by means of an enzyme, with the production of ammonia. Nadson (1903) stated that the Actinomycetes isolated from the curative mud of the Veisov salt lake produce a precipitate of the casein of the milk. The clot is then dissolved gradually from the surface downward and the milk is transformed into an almost transparent fluid, having a reddish opalescence; the liquid is distinctly alkaline and a small portion of undissolved casein is left at the bottom of the tube.

A preliminary paper was recently published by the writer (1918) on the metabolism of pathogenic Actinomycetes, in which the question of the action of these organisms upon milk was discussed, but since it was brief and preliminary the results will be included in table 1 for comparative purposes.

Experimental

The organisms used in this work were isolated from the soil in the end of 1915 by Waksman and Curtis and briefly described in 1916. *A. madurae*, *A. hominis*, *A. bovis*, *A. asteroides* and *A. scabies* were obtained from sources noted above. A full description of the organisms indicated by numbers as well as of those previously described will appear later. All the organisms were grown upon a synthetic medium,[1] with the exception of the four animal pathogens.

Freshly separated milk was placed in tubes and flasks and sterilized for thirty minutes on three consecutive days in flowing steam at 100°C. The sterile milk was then inoculated from the different cultures grown on the synthetic medium and incubated at 37°C. At the end of the incubation period amino nitrogen and ammonia were determined in some of the cultures, the amino nitrogen by the use of the micro-apparatus of Van Slyke (1914); the ammonia by the Folin aeration method, as modified by Steele (1910).

The indicator suggested by Clark and Lubs (1917), dibrom-orthocresolsulfonphthalein, or more simply "bromcresol purple," was found to be excellently suited for the study of reaction changes in milk and was used in place of litmus for this purpose.

The indicator was dissolved in NaOH solution and added to the fresh, unadjusted milk in the proportions suggested by Clark and Lubs (1917). Since it is very difficult to describe the color changes of the indicator in solution and we have no standards for determining colorimetrically the P_H values of milk satisfactorily, the changes in reaction produced as a result of the growth of the Actinomycetes on milk are indicated as follows: 0 designates no change in reaction (about P_H 6.3); − acid; + weakly alkaline; ++, +++, ++++ designate different degrees of alkalinity, the last being the most alkaline cultures as indicated by the use of the particular indicator.

The results brought out in table 1 point defin tely to the fact that the chemical changes produced in milk due to the action of Actinomycetes can be taken advantage of in the identification of the species of these organisms. The chemical changes of the milk constituents seem to be quite definite for different species of Actinomycetes. The results brought out in the above table were duplicated several times and where discrepancies occurred attention will be called to them in the following discussion.

First we find several organisms, such as *A. griseus*, *A. poolensis*, *A. madurae*, 206, 214, 215, which coagulate the milk in three to five days, soon begin to peptonize the curd, and have digested it all in about ten days. The amount of amino nitrogen and ammonia produced by these organisms is very great; for example,

TABLE 1

*The action of Actinomycetes upon milk**

NAME OF ORGANISM	COAGULATION	PEPTONIZATION			HYDROLYSIS	NO ACTION	BROWN PIGMENT	NH_3-N IN 15 DAYS		NH_3-N IN 40 DAYS		NH_3-N IN 40 DAYS		REACTION§
		Start	End	Rapidity‡				Milligrams in 10 cc.	Per cent	Milligrams in 10 cc.	Per cent	Milligrams in 10 cc.	Per cent	
	days	*days*	*days*	*days*										
Control (unincubated)	–	–	–	–	–	–	–	1.33	2.2	1.33	2.2	0.5	0.84	Unadjusted
Control (incubated)	–	–	–	–	–	–	–			2.65	4.4	0.5	0.84	0
A. aureus	–	–	–	–	–	+	+	5.11	8.6	14.82	24.9	7.2	12.1	0
A. griseus	4–5	5	10	++++	–	–	–	34.40	57.7	33.21	54.0	13.9	23.3	++++
A. californicus	15	15	Not in 45	+	–	–	–	4.56	7.6					+
A. viridochromogenus†	5–6	6	12	+++	–	–	–	6.80	11.5	17.96	30.1	4.3	7.2	+
A. albus	–	–	–	–	20	–	–	9.12	15.4	23.66	39.7	11.0	18.4	++++
A. diastaticus	5–7	7	18–25	++	–	–	–	8.76	14.7	25.94	43.5	10.1	16.9	+
A. fradii†	10–12	12	20	++	–	–	–	5.69	9.5	19.95	33.5	5.3	8.9	++
A. violaceus-ruber	–	–	–	–	12–15	–	–			21.38	35.9	9.6	16.1	+++
A. violaceus-caesari	10–12	12	50	+	–	–	–			20.80	34.9	6.5	10.9	+
A. purpeo-chromogenus	10	10	Not in 50	+	–	–	–			13.45	23.4	3.4	5.6	+
A. exfoliatus	–	–	–	–	8–10	–	–			15.96	26.8	9.1	15.3	++++
A. reticuli	4–6	6	Not 50	+	–	–	+			14.25	23.9	6.5	10.9	0
A. 120	4–6	6	Not 50	+	–	–	+	7.10	11.9	16.18	27.1			0
A. citreus†	9–10	10	20	+++	–	–	–			25.94	43.5	12.7	21.3	++
A. alboflavus	–	–	–	–	10–12	–	–			26.32	44.2	7.7	12.9	+++
A. verne	4–5	5	18	+++	–	–	–			28.50	47.8	9.6	16.1	++
A. albosporeus	–	–	–	–	–	+	–			9.41	15.8	1.9	3.2	0
A. bobili	–	–	–	–	15–18	–	–			26.79	44.9	10.8	18.1	+++
A. lipmanii	8–9	9	30	++	–	–	–	5.8	9.7	23.37	39.2	4.9	8.3	++
A. rutgersensis	5–6	6	Not 50	+	–	–	–			22.80	38.3	5.8	9.7	++
A. roseus	–	–	–	–	10–15	–	–	4.30	7.2	11.69	19.6	6.7	11.2	+++

A. lavendulae	–	–	–	–	20–30	–	+			15.68	26.3	5.8	9.7	++++
A. madurae	3–4	3–4	10–20	+++	–	–	–	35.63	59.8					+
A. hominis	5–6	5–6	20	+++	–	–	–	19.95	33.5					+++
A. bovis†	10–12	12	40	++	–	–	–	9.98	16.8					+
A. asteroides	–	–	–	–	–	+	–	5.60	9.6					0
A. halstedii	10	10	Not 50	+	–	–	–			11.69	19.9	1.9	3.2	++
A. poolensis†	4–5	4–5	10	++++	–	–	–	20.60	34.6	30.21	50.7	13.0	21.8	++++
A. scabies I†	5–10	5–10	10–30	++	–	–	+			12.83	21.5	9.1	15.3	++
A. scabies II	10–12	12	15–30	++	–	–	+			9.22	15.5			++
A. pheochromogenus	–	–	–	–	–	+	+	4.85	8.1	5.70	9.6	2.2	3.7	+
	(soft clot often)										*			
214	3–4	3–4	8–10	++++	–	–	–			31.64	53.1	14.4	24.1	+++
215	3–4	3–4	7–10	++++	–	–	–			21.95	36.8	12.0	20.1	++
96	10–20	10–20	30	++	–	–	–			21.95	36.8	11.8	19.8	++
128	3–6	3–6	15–30	++	–	–	–	7.89	13.2	25.65	43.0	12.7	21.3	+
145	6–7	7	Not 50	+	–	–	–	5.40	9.0	5.70	9.6	3.5	5.9	0
154	4–5	5–6	Not 50	+	–	–	–			23.66	39.7	6.7	11.2	0
161	6–7	7–8	Not 50	+	–	–	–			21.66	36.3	10.8	18.1	+++
168†	5–8	6–8	20	++	–	–	–			16.96	28.5	13.2	22.2	+
190	4–6	5–6	15–30	++	–	–	–			31.92	54.2	13.9	23.3	+++
198†	5–6	5–6	18	+++	–	–	+			16.53	27.7	4.8	8.0	+++
202	–	–	–	–	–	+	+			10.55	17.7	5.3	8.9	0
205	–	–	–	–	–	+	+			13.12	22.0	4.3	7.2	+++
206	4–5	4–5	10–12	++++	–	–	–			28.32	47.5	13.7	23.0	++
B. subtilis	2	2	6–8	++++	–	–	–			18.81	31.6	15.4	25.8	++++
B. mycoides	2	2	6–8	++++	–	–	–			21.09	35.4	19.0	31.9	++++
B. coli	6	0	–	–	–	–	–			3.14	5.3	1.7	2.8	acid

*Total nitrogen in 10 cc. of milk is 59.6 mgm.

†Hydrolized also in one or more cases.

‡– Negative, + weak, ++ fair, +++ rapid, ++++ very rapid.

§ 0 designates no change in reaction, +, ++, +++, ++++ designate different degrees of alkalinity, ++++ being the most alkaline reaction.

A. griseus converted in fifteen days 57.7 per cent, *A. madurae* 59.8 per cent and *A. poolensis* 34.6 per cent of the nitrogen of the milk into amino-nitrogen. *A. griseus* converted 23.3, *A. poolensis* 21.8, 214–24.1, 215–20.1, 206–23.0 per cent of the nitrogen of the milk into ammonia in forty days.

The reaction of the milk becomes highly alkaline in this series of organisms; this alkalinity is no doubt due to the production of large quantities of ammonia and other basic substances and possibly to the production of carbonates from the milk sugars.

The second group of organisms contains those species that coagulate the milk rapidly, but peptonize the curd slowly, so that in most cases the curd is not digested even in forty-five days. In this group of organisms we would include *A. reticuli*, 43 and 120, *A. rutgersensis*, 145, 154, 161. If the rennin and the proteolytic enzymes are distinct, it would seem that these organisms produce as strong a rennet-like enzyme as the first group, but a weaker proteolytic enzyme, which therefore accounts for the slow peptonization of the curd. This group of organisms is further characterized, in general, by the production of a low alkalinity, particularly in the case of the *A. reticuli* cultures and 145 which produced almost no change in the reaction of the milk. This fact is in accord with the assumption that the production of a proteolytic enzyme by these organisms is limited.

Several organisms, namely *A. hominis*, 128, *A. viridochromogenus*, *A. diastaticus*, *A. verne*, stand between the two groups, producing a rapid coagulation, the peptonization being more slow than in group I and more rapid than in group II. Since this division has no sharp lines of demarcation and is purely artificial, the intermediate organisms can be considered as related to both of these groups.

Those organisms which coagulate the milk only in ten to twelve days followed by a rather rapid peptonization would form a third group. Here we would include such organisms as *A. fradii*, *A. lipmanii*, *A. bovis*, *A. scabies*, 96, *A. citreus* and 168. These organisms are characterized by an alkalinity lower than group I but higher than group II, no doubt due to the fairly rapid digestion of the clot.

A fourth group would comprise those organisms which do not coagulate the milk, but hydrolize it without previous coagulation. Here we would include *A. albus*, *A. exfoliatus*, *A. violaceus-ruber*, *A. bobili*, *A. lavendulae*, *A. roseus* and *A. alboflavus*. These are all characterized by a very high alkalinity. It is possible that the lack of coagulation is not due to the lack of a rennet-like enzyme, but merely to the fact that the digestion of the casein of the milk proceeds rapidly at its early stages, so that coagulation is not observed or is absent. That some of the organisms that coagulate the milk may produce hydrolysis without clotting can also be concluded from the fact that a few cultures, namely, *A. fradii*, *A. bovis*, *A. scabies*, *A. citreus* and 198 produced hydrolysis of the milk in some cases, while in other cases the same organisms produced a clot and then digested it. This will explain the fact that *A. bovis* and *A. scabies* (*A. chromogenus*) are able, as reported by some investigators, to coagulate the milk and then peptonize it, while others state that these organisms can only hydrolize the milk without previously coagulating it. It is interesting to note that all these organisms which hydrolized the milk without previously clotting it at one time or produced a clot and then digested it at another time, were placed in group III, which contains the organisms that produce a clot late but peptonize the clot rapidly. The possible explanations would be either that these organisms produce rennin at one time and not at another, which is hardly probable, or that the clot formation and the peptonization of the casein go hand in hand so that coagulation is often not observed. The interaction between casein and the proteolytic enzyme may be more rapid than between casein and rennin. The lack of clot may therefore not necessarily indicate the lack of production of a rennet-like enzyme, as will be pointed out again in another connection.

The fifth group consisted of strains which seemed to have no effect upon the milk in thirty days. Some of these organisms are probably unable to grow in milk and could not be reisolated; *A. aureus*, *A. pheochromogenus*, 202, 205 belong to this group. In the case of the organisms that did grow upon the milk, such as *A. asteroides*, the action upon the casein is so slight as to

produce no visible change. These organisms seem to produce a very weak proteolytic enzyme and no rennet action; a longer incubation period (forty-five days) will indicate a degree of fair digestion of the milk protein.

The species belonging to this group are mostly chromogenus types, i.e., producing black pigments on organic media. When grown on milk at 25°C., all of them produced a surface brown to black ring, accompanied by an imperfect clot formation and weak digestion; the animo nitrogen content of the milk was rather low and the reaction quite alkaline (++ or +++).

In general when grown at 25°, nearly all the species produced a better growth upon the milk, but the action upon the milk, ran about alike to that obtained at 37°.

The Actinomycetes are variable in nature and the amount of growth upon different media depends upon many factors, such as mother cultures, temperature of incubation, etc. We would therefore expect that, at different temperatures of incubation, some species at least, would show distinctive differences in the clot formation, rapidity of peptinization and change of reaction. These differences were actually obtained but they varied in degree rather than in kind.

The division of the Actinomycetes into five groups according to their action upon the milk constituents should be looked upon as only an attempt to point out in a definite manner a possible differentiation of these organisms. Although, upon repeated transfer, the same reactions in milk were generally obtained, there were several exceptions and therefore the division lines cannot be absolute. The production by some organisms of a clot at one time and hydrolysis of the milk, without previous clotting, at another has already been pointed out.

A. madurae produced a clot in three to four days and then digested it rapidly; but, when grown for a long time upon synthetic media, it clotted the milk in about the same period of time but digested it slowly. This may perhaps be due to the nature of the medium upon which the organism has been grown previously. Such an effect of acclimatization upon the biochemical activities of microorganisms has not been studied in detail as

yet, although we know that it is important. For example, it is known that the production of enzymes by microorganisms depends upon the nature of the medium upon which they are grown, but we know also that this difference is of a quantitative rather than a qualitative nature. Hardly any attention has ever been paid to the influence of the medium on which an organism was grown, upon the production of enzymes by this organism, when transferred into a new medium. When more information on this subject has been accumulated, some of the discrepancies observed in the growth of Actinomycetes on milk, which are doubtless enzymatic phenomena, will probably be explained.

The change of the reaction of the milk is very characteristic. In nearly all cases the milk became alkaline; in only a few instances did the reaction fail to change; in no instance did the reaction become acid. The amount of alkalinity seems to go hand in hand with the amount of digestion. When the coagulum was produced there was usually no change or very little change of reaction; when the digestion began, the reaction became more alkaline and continued to increase with the advance of the digestion, proceeding from surface to bottom.

When the two periods of incubation are compared, it becomes evident that greater differentiation can be observed for the different cultures, if a record is taken at the end of a shorter period of incubation, namely about fifteen days at 37°. Since most of the organisms are active proteolytically and are able to digest the milk proteins, a long incubation period will tend towards the elimination of the differences, as observed by inspection of the cultures. For example, *A. griseus*, one of the most active Actinomycetes studied, almost completely digested the milk proteins in fifteen days, producing 57.7 per cent amino nitrogen and about 20 per cent ammonia nitrogen, while at forty-five days these quantities were changed to 54.0 and 23.3 per cent. This would indicate that very little splitting of proteins took place between the two incubation periods, and that some amino nitrogen compounds were reduced to ammonia. On the other hand the cultures of weaker proteolytic organisms, such as *A. diastaticus* and *A. fradii*, which contained at the end of fifteen days only 14.7

and 9.5 per cent amino nitrogen, have shown in forty-five days an increase to 43.5 and 33.5 per cent respectively.

The comparatively large quantities of amino nitrogen and ammonia produced by some Actinomycetes from milk proteins would indicate that some of these organisms may be very important in the splitting of proteins in nature. Their activity becomes more evident when compared with the action of several common bacteria. *B. mycoides* and *B. subtilis*, which are among the most active proteolytic bacteria known, produced from the milk proteins, with the same period and temperature of incubation, less amino nitrogen and only slightly larger quantities of ammonia nitrogen than the Actinomycetes placed in group I; *B. coli*, which is known as a weak proteolytic organism, produced an acid clot, and split the milk proteins only to a very limited extent.

To obtain further information in regard to the action of the Actinomycetes upon the milk and to determine whether the phenomena observed were due to enzyme activities or to the growth of the cells, the following experiments were carried out:

One cubic centimeter of the fifteen-day milk culture of the organisms (in case the coagulum was partly or fully digested, the whey was used) was added to 10 cc. of fresh sterile milk in test tubes, using about 1 cc. of toluene to prevent any growth from taking place.

To test also the proteolytic action of the culture, 1 cc. of the fluid was added to 10 cc. of sterile gelatin, 15 per cent in distilled water, using 1 cc. of toluene for each tube. The tubes were then stoppered with rubber stoppers and incubated at 37°C. For examination, the gelatin cultures were placed for one hour on ice, then examined; if they remained liquid, they were pronounced digested and if they solidified again, undigested.

The results are given in table 2.

The production of a rennet-like and proteolytic enzyme is definite, although we might argue from the data presented in table 2 that both activities can be attributed to the same enzyme, and that the difference between the proteolytic and rennet-like action depends entirely upon the velocity of the action of the enzyme.

For example, *A. griseus*, which seems to possess a strong proteolytic enzyme, and which always clotted the milk very rapidly, did not produce any clot when only the enzyme was studied, but rapidly peptonized (hydrolized) the milk and liquefied the gelatin. So much of the proteolytic enzyme had been added that the digestion of the casein of the milk proceeded very rapidly and no clotting was observed. In the case of the other organisms which possess less active proteolytic enzymes, as indicated by

TABLE 2

The production of rennet-like and proteolytic enzymes by Actinomycetes

NAME OF ORGANISM	COAGULATION OF MILK	PEPTONIZATION OF COAGULUM	HYDROLYSIS	LIQUEFACTION OF GELATIN
	days	*days*	*days*	*days*
A. griseus	—	—	2	1
A. griseus	1	4	—	1
A. diastaticus	3	10	—	3
A. lipmanii	4	Slow	—	5
A. californicus	4	Slow	—	5
A. albus	—	—	—	5
A. viridochromogenus	4	Slow	—	2
A. poolensis	3	8–10	—	Not determined
A. citreus	2	7–10	—	Not determined
96	—	—	6	Not determined
A. scabies	—	—	—	—
A. pheochromogenus	—	—	—	—
A. aureus	—	—	—	—
A. reticuli	—	—	—	—
A. exfoliatus	—	—	15	Not determined
198	12	30	—	Not determined
215	4	8–10	—	3

the period of time necessary for the liquefaction of the gelatin and the peptonization of the milk, the milk was clotted. Organism 96, which clotted the milk very slowly (see table 1), did not show any rennet-like enzyme although peptonization of the milk (hydrolysis) took place. This may add weight to the assumption that the rennet-like and proteolytic enzymes are one and the same thing, the rennet-like action being merely the first step in the action of the proteolytic enzyme, but when the enzyme is

either too active, as in the case of *A. griseus*, or too inactive, as in the case of 96, no clotting of the milk will be observed. The organisms that produced no visible action upon the milk, or acted upon it very slowly, did not exhibit any active proteolytic enzymes.

To obtain a more or less pure enzyme for work, *A. griseus*, *A. exfoliatus* and *A. lipmanii* were grown in 100 cc. quantities of sterile milk in 200 cc. Erlenmeyer flasks for twenty days. At the end of that period, *A. griseus* and *A. exfoliatus* cultures were wholly and *A. lipmanii* cultures largely peptonized. The clear filtered fluid was precipitated by means of 95 per cent alcohol; the precipitate was washed with absolute alcohol and ether, and dried over sulfuric acid. The dried material was added (about 10 mgm.) to 10 cc. of sterile milk and incubated at 37°; toluene was used to prevent contamination. The enzymes of the three organisms produced a complete clot of the milk in twenty-four hours. The clot was not digested readily even after ten to twelve days incubation at 37°. A proteolytic and rennet-like enzyme is therefore shown to be certainly present in some species of Actinomycetes.

The last experiment would lead to the assumption that the rennet-like enzyme can thus be shown to be distinctly different from the proteolytic enzyme. *A. griseus* and *A. exfoliatus* were grown again in 300 cc. quantities of sterile milk in 1 liter Erlenmeyer flasks till all the milk was digested (*A. griseus* first produced a clot in six to seven days with perfect digestion in twelve to fifteen days; *A. exfoliatus* hydrolized the milk in fifteen days). The milk was filtered through paper and the filtrate precipitated by means of alcohol, redissolved in water and reprecipitated; then washed with absolute alcohol and ether and dried over sulfuric acid. The material was scraped off the paper and added to a series of tubes of milk (about 5 to 10 mgm. to 10 cc. of milk). A perfect clot was produced in all cases without any whey formation for the *A. exfoliatus* and only a small quantity (0.5 to 0.75 cc.) for the *A. griseus* in ten to fifteen days at 37°. The whey of the latter contained 4.03 mgm. amino nitrogen per 10 cc. of whey, indicating a very faint digestion which is no doubt

due to a trace of the proteolytic enzyme precipitating with the rennet-like enzyme. It is interesting to note that *A. exfoliatus* which, when grown on milk, produced no clot but merely hydrolized the milk gave a strong rennet-like enzyme.

Several Actinomycetes were grown in Czapek's synthetic solution for thirty days in Erlenmeyer flasks at 22°. At the end of that period the cultures were filtered and both filtrate and mycelium used for study of the presence of rennet-like and proteolytic enzymes, the former containing the exoenzymes and the latter the endoenzymes. The mycelium was washed in water several times, then treated by the "acetone-dauerhefe" method (ten minutes in acetone, three minutes in ether and dried over sulfuric acid).

One cubic centimeter of the filtrate and 25 mgm. of the treated mycelium were added to test tubes containing 10 cc. of sterile milk, using toluene to prevent contamination. The tubes were incubated at 37°. The results are given in table 3.

The data presented in table 3 point to the fact that both types of active proteolytic enzymes are produced by Actinomycetes when grown on synthetic media. Several interesting observations can be made from these data. The exoenzyme (filtrate) has very active rennetic properties, as shown by the formation of a clot, and rather weak proteolytic properties, as shown by the fact that either the coagulum was not digested, as in the case of *A. aureus* and 168, or was digested only slowly (*A. fradii*) or to a very limited extent. The endoenzyme (mycelium) contained a weak rennet-like enzyme or none at all, but a very active proteolytic enzyme, as shown by the amino nitrogen in the medium. It is interesting to note that *A. aureus*, which did not produce any rennet-like endoenzyme, does not clot the milk when grown upon that medium, and 168, the ectoenzyme of which did not clot the milk, but hydrolized it, often produces a hydrolysis of the milk when grown upon that medium.

It would look as if the rennet-like enzyme is dissolved out into the medium, while the proteolytic enzyme is kept largely within the mycelium, this tending to indicate a distinct difference between the two enzymes.

TABLE 3

The production of rennet-like and proteolytic enzymes by Actinomycetes grown upon synthetic media

NAME OF ORGANISM		EXOENZYME				ENDOENZYME		
		Number of cubic centimeters	Coagulation days	Digestion days	NH_3–N in 10 cc.	Coagulation days	Digestion days	NH_3–N in 10 cc.
A. diastaticus		1	4	Trace	–	4	½ in 15	9.7
		2	4	Trace	–			
		3	2	½ in 15	7.3			
A. aureus		1	15	Trace	–	0	0	5.9
		2	12	Trace	–			
		3	4	Trace	–			
A. fradii		1	2	Complete in 15–30	11.7	3	10	25.0
		2	2	Complete in 15–30	–			
		3	1	Complete in 15–30	–			
168		1	4	Trace	–	0	Hydrolysis	24.5
		2	2	Trace	–			
		3	2	Trace	–			

SUMMARY

1. The Actinomycetes vary greatly in their action upon milk.

2. These organisms can be divided into five groups, using as a basis their action upon milk, although no sharp lines can be drawn between the different groups which blend into one another: group I contains those organisms that coagulate the milk rapidly and then peptonize the coagulum rapidly; group II, organisms that coagulate the milk rapidly, but peptonize the coagulum slowly; group III, those organisms which coagulate the milk slowly, but then peptonize the coagulum fairly rapidly; the organisms that peptonize (hydrolize) the milk, without coagulating it, will form group IV; and group V includes those organisms that have no visible action upon the milk. The differences will

be observed more readily when the period of incubation at 37°C. is not too long.

3. A few species do not lend themselves readily to this system of classification and are to be grouped in positions intermediate between two of these groups.

4. A few species give variable results on repeated inoculation in milk; this variability can be explained when the metabolism of the proper organisms is taken into consideration.

5. Rennet-like and active proteolytic enzymes are produced by the organisms that exert a strong proteolytic action upon the milk.

6. The reaction of the milk is changed in nearly all cases to an alkaline one.

7. The rennet-like and proteolytic enzymes, produced by the Actinomycetes, seem to be distinct from one another.

III. THE USE OF BLOOD MEDIA FOR THE STUDY OF ACTINOMYCETES[2]

The Actinomycetes were studied on blood media for two reasons: firstly, to find out whether these media, which are well adapted for the growth of most pathogenic bacteria, can also be used successfully for the study of Actinomycetes; secondly, to see whether growth on these media brings about characteristic reactions, which indicate the metabolism of these organisms and help in their proper identification.

Schlegel (1913) states that Israel and Kischevsky found coagulated serum to be a good medium for the growth of Actinomycetes. *A. bovis* was reported by some investigators to grow well on blood serum with the liquefaction of the serum, and *A. madurae* was reported not to produce any growth on blood serum. Goadby (1903) stated that *Streptothrix buccalis* isolated from the mouth grew on blood serum (at 37.5°C.) and liquefaction of the serum was noted. Macé (1905) stated that *Cladothrix* (*Actinomyces*) develops well in liquid blood serum, producing a browning of the medium and a characteristic odor. After several months, the

[2] The work included in this paper was partly done by the writer at the Cutter Biological Laboratories, Berkeley, Cal.

liquid becomes more fluid and, while it does not coagulate upon heating, gives a slight floccose precipitate on boiling. The liquid contains ammonia, pro-peptones, crystals of tyrosin, leucin and glycocoll, but no indol. This species was isolated by Macé from the soil and was found to be one of the active agents in the transformation of albuminous matter. The writer (1918) has recently pointed out that some pathogenic Actinomycetes can liquefy blood serum and produce hemolysis of whole blood medium, while others do not; the Actinomycetes seem to vary markedly in this respect.

These observations would seem to point out the fact that at least some Actinomycetes are active proteolytically when blood proteins are present as a substratum.

Both whole blood and blood serum were used for this work. The blood agar was prepared as follows: nutrient agar containing veal infusion (500 grams of veal per liter of tap water, boiled ten minutes and filtered), 1 per cent Bacto-peptone, 0.5 per cent sodium chloride, 2.5 per cent agar, adjusted to + 1.0 (P_H = 7.6–7.8) flasked and sterilized. After sterilization, the agar was cooled to 45 to 50°C. and about 10 per cent of sterile rabbit blood was added, the flask was well shaken, and the agar poured, under sterile conditions, into sterile test-tubes or sterile Petri dishes; the tubes were slanted, and both tubes and dishes incubated for forty-eight hours to insure sterility.

The Loeffler's blood serum was prepared according to the usual formula: to 3 parts of ox-blood serum 1 part of glucose bouillon was added containing veal or beef infusion prepared as before, 1 per cent each of peptone and glucose and 0.5 per cent sodium chloride, reaction + 1.0 (P_H 7.6 to 7.8); these were well mixed, passed through a Berkefeld filter, tubed into sterile tubes, using sterile containers, slanted in the Arnold, coagulated and sterilized, the process lasting for two hours, the temperature never going up above 90°C. The tubes were incubated for forty-eight hours to insure sterility.

Growth of Actinomycetes on blood agar

The organisms grown upon the synthetic medium (Czapek's) were inoculated upon the blood agar, tubes and plates, and cultures incubated at 37°C. In nearly all instances a good growth was obtained in twenty-four to forty-eight hours, with the exception of *A. pheochromegenus* which grew very slowly; blood agar forms, therefore, a good medium for the growth of the Actinomycetes.

TABLE 4

The growth of Actinomycetes upon blood agar and the production of hemolysis

NAME OF ORGANISM	COLOR OF COLONY	SOLUBLE PIGMENT	AERIAL MYCELIUM	HEMOLYSIS
A. aureus	Dark brown	Dark	–	0
A. albus	Green	–	White	0
A. griseus	Greenish	–	White	+++
A. diastaticus	Grayish brown	–	–	++
A. californicus	Red to gray	–	–	0
A. poolensis	Green	–	–	0
A. fradii	Brown	–	–	0
A. lipmanii	Greenish	–	–	0
A. viridochromogenus	Dark brown	Dark	Scant white	0
A. scabies	Brownish to gray	Dark	–	0
A. pheochromogenus	Brown	–	–	0
215	Green	Dark gray	–	+
128	Green	–	White	+++
168	Brownish	–	White	+
A. citreus	Gray	–	–	0
A. bovis				+
A. hominis				++
A. asteroides				0
A. madurae				+++

The data brought out in table 4 tend to indicate that although some Actinomycetes show characteristic reactions upon blood agar, such as *A. griseus*, *A. madurae* and 128, the different species could not be readily differentiated by this medium alone. The cultural characters on blood agar can be used only to a very limited extent for the differentiation of these organisms. The interesting fact brought out in table 4 is the hemolysis of the blood in the blood agar, either as a result of the growth of the

organism or as a result of the production of an enzyme-like hemolysin. In the case of the organism producing hemolysis, a clear zone is formed around the colony on the plate or tube. In the case of *A. griseus*, *A. madurae* and *A. diastaticus* this zone was already observed in twenty-four hours.

Kuhn (1912) claimed that hemolysis is a step towards virulence, although not identical with virulence; the hemolysis of an organism is a sign of a higher step of adjustment of the organism to a parasitic (animal) existence. If this were the case for all microorganisms, the Actinomycetes isolated from the soil would be closely related to the parasitic Actinomycetes not only morphologically, but also physiologically which no doubt holds true, as will be seen throughout the work reported in these papers. But still another assumption can be made here. Several veterinarians expressed to the writer their opinion that, in certain localities, cattle feeding mainly on grass suffer quite often from outbreaks of actinomycotic diseases, and that this disease is obtained from the soil. Certain soils, as will be pointed out elsewhere, contain as many as 40 per cent Actinomycetes out of the total microbial population obtained by plate culture counts. The possibility of some of these Actinomycetes being able to produce animal diseases may be therefore not without foundation, although it has not been demonstrated as yet.

As to the chemical changes involved in the action of Actinomycetes upon blood, we might refer here to a paper by Baerthlein (1914) who stated that among the modifications which blood undergoes due to the action of bacteria, one can distinguish: (1) Hemolysis proper; the hemoglobin is set free and is not modified, the corpuscular stroma remains intact. (2) Hemoglobinopepsie; hemoglobin is set free and is completely digested, which brings about a discoloration of the medium, the corpuscular stroma remains intact. (3) Hemopepsie; the medium is decolorized as in the previous case, but the corpuscular stroma is digested as well as the hemoglobin. Cases 2 and 3 are observed only on solid media, while hemolysis proper is observed in liquid media. The use of the term "hemolysis" should therefore be

understood to include not only hemolysis proper, but also the digestion of the hemoglobin. Only hemoglobin, when heated or treated with acids, alkalies or various digestive enzymes, forms hematin, which has a brown appearance. It is possible that the action upon blood of those organisms. which do not produce any clear zone, but a brown pigment, is due to the fact that an enzyme is produced which converts the hemoglobin to hematin and related dark brown compounds, while the organisms forming a clear zone produce enzymes which split the hemoglobin molecule entirely liberating the iron or absorbing it. McNeal and Kahn (1918) have recently stated that hemolysis and proteolysis are probably two distinct phenomena. This is not borne out by the work on the Actinomycetes, since of the organisms that were isolated as saprophytes (from the soil) the ones that are most active proteolytically are also able to produce hemolysis of the blood in blood agar.

Growth of Actinomycetes on Loeffler's blood serum

Methods of inoculation and incubation were the same as for the blood agar. The results are reported in table 5.

Most of the Actinomycetes do not produce any characteristic growth on this medium, although all of them, with few exceptions, grew very well upon it. The liquefaction of the coagulated serum is an important biochemical property of some species of Actinomycetes. It will be noted that those organisms that were active in producing extensive hemolysis produced also liquefaction of the serum. These very strains were among the most active proteolytically, when grown on milk. It can thus be seen that the organisms that are able to decompose the casein of the milk, with the production of a strong proteolytic enzyme, also liquefy the blood serum, which is also a proteolytic phenomenon, and produce hemolysis of the blood in the blood serum.

To demonstrate that the liquefaction of the serum is a proteolytic phenomenon and is accompanied by a decomposition of the serum proteins, some of the liquefied serum was withdrawn from the culture and the amino nitrogen determined by the use

of the Van Slyke apparatus. *A. griseus* gave 18.10 mgm. amino nitrogen per 10 cc. and 128 gave 22.33 mgm. The serum originally contained 4.56 mgm. amino nitrogen per 10 cc. This indicates that the liquefaction of the serum was accompanied by the splitting of the serum proteins into peptones, polypeptides and probably amino acids and ammonia.

TABLE 5

The growth of Actinomycetes upon Loeffler's blood serum

NAME OF ORGANISM	COLOR OF GROWTH	SOLUBLE PIGMENT	AERIAL MYCELIUM	LIQUEFACTION TWO MONTHS
A. violaceus-ruber	Brown to red	Red	–	0
A. violaceus-Caesari	Gray	–	–	0
A. albus	Transparent	–	–	0
A. griseus	Creamy	–	White	++++
A. diastaticus	Transparent	–	–	++
A. californicus	Brown	–	–	0
A. aureus	Creamy	Black	–	0
A. poolensis	Creamy	–	–	0
A. fradii	Orange	–	–	0
A. lavendulae	White	Purple	–	0
A. lipmanii	Transparent	–	–	++
A. viridochromogenus	Brown	Black	White	0
A. scabies	Gray to somewhat yellowish	Brown	–	0
A. rutgersensis	Gray	–	–	0
A. reticuli	Gray	Brown	–	0
A. 120	Gray	Brown	–	0
A. bibili	Gray	Brown	–	0
A. citreus	Creamy	–	–	0
A. bovis	Yellowish	–	White	++
A. hominus	Transparent	–	–	+++
A. asteroides	Thin white	–	–	0
A. madurae	Yellowish	–	White	+++
128	Creamy	–	White	+++
168	Light brown	–	–	+
215	Sulfur-yellow	Black	–	0
206				++

SUMMARY

1. Blood agar forms a good medium for the growth of Actinomycetes. The production of a dark pigment by some and a clear zone, indicating hemolysis, by others is characteristic.

2. Loeffler's blood serum forms a good medium for the growth of Actinomycetes; the liquefaction of the serum by some and the production of a dark brown to black pigment by others are distinctive properties of some of these organisms.

3. The organisms that produce hemolysis and liquefy the blood serum are among the ones that can produce active proteolytic enzymes.

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STUDIES ON DIPHTHERIA TOXIN[1]

II. THE RÔLE OF THE AMINO ACIDS IN THE METABOLISM OF BACTERIUM DIPHTHERIAE

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Attempts to cultivate *Bact. diphtheriae* in simple, non-protein media are recorded among the early studies with this organism. In 1892, Guinochet (1892) claimed to have grown diphtheria bacilli with toxin production in urine freed from albuminous constituents. Soon after, Uschinsky (1893) confirmed these findings and also reported the growth of *Bact. diphtheriae* with elaboration of toxin in a medium having ammonium lactate and sodium asparaginate as the sole sources of nitrogen. The toxin thus obtained gave positive Millon's and xanthoproteic reactions, was precipitable by alcohol, and in its other reactions indicated protein characteristics.

The view that diphtheria toxin is a form of protein seems to have been first advocated by Brieger and Fraenkel (1890). These authors considered it to be a peculiar kind of albumin, which, because of its toxicity, they called "toxalbumin." Wasserman and Proskauer (1891), while conceding the possibility that pure diphtheria toxin may be an albuminous body, were of the opinion that the chemical reactions of the above "toxalbumin" were probably due to albumoses, present as impurities derived from bouillon. They also agreed with the observations of Brieger and Fraenkel that, because of its great susceptibility, this "toxalbumin" of diphtheria contained only a small portion of actual diphtheria toxin in an undecomposed state.

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A unique view regarding the composition of diphtheria toxin was that advanced by Dzierzgowski and Rekowski (1892). From the fact that no diphtheria toxin was formed until the culture became alkaline, these investigators concluded that diphtheria toxin was a combination of certain bases and albumoses, of a nature similar to alkali albuminates.

Brieger and Boer (1896) working with both diphtheria and tetanus toxins, claimed to have isolated a double zinc-toxin compound, which showed neither albumin nor peptone reactions, thus arguing against the protein character of diphtheria toxin. In a subsequent communication regarding his synthetic diphtheria toxin, Uschinsky (1897) did not consider it of albuminous nature, although Chamberland filtrates of diphtheria bacilli grown in his medium gave distinct protein reactions. Not all cultures of *Bact. diphtheriae* were found by him to be cultivable in a protein free medium, but when once accustomed to the medium, the organisms grew typically and as luxuriantly as in bouillon.

In an elaborate investigation comprehending seventy pure cultures of *Bact. diphtheriae*, Hadley (1907) was unable to obtain any noticeable growth on Uschinsky's medium. Continuing the experimentation, this investigator finally devised a medium containing glycocoll and ammonium lactate together with glycerin, sodium chloride, calcium chloride, magnesium sulphate and di-potassium phosphate. This he found not only permitted the growth of diphtheria bacilli, but produced a culture, which after sixteen days' incubation, killed guinea pigs in from thirty-six to thirty-eight hours. Cultures of *Bact. diphtheriae* which, at first, did not grow on the protein-free media, could be adapted to it by slow degrees and the solid-staining forms of the organism always manifested the most rapid and luxuriant growth both during and after the adaptation. Hadley further concluded that, of the three nitrogen bases employed in the study, urea seemed to be of slight value, while glycocoll gave the best growth and strongest toxin. Asparagin appeared to give better results than the urea although it was not as satisfactory as the glycocoll.

Hida (1908), in a paper concerning the significance of "pep-

tone" in the formation of diphtheria toxin concludes that the deutero-albumose portion of the peptone is the most important for this purpose, while heteroalbumose, protalbumose, and amphopeptone are subordinate factors. A subsequent article by the same author working with Teruuchi (1912–1913) also indicated that the production of toxin was influenced by the degree of decomposition of the "peptone." Most favorable results were furnished by a sample having 18 per cent of its total nitrogen in the monamino acid condition, 75 per cent, precipitable by tannin, and 30 per cent by a 65 per cent concentration of alcohol.

The chemical composition of diphtheria bacilli has been reported upon by Tamura (1914) who examined, washed and dried pellicles of the organisms cultivated in a 2 per cent peptone, sheep-kidney extract medium. A monoaminomonophosphatid and a lipoid body were found to be present and the presence of adenin was considered as probable. Of the amino acids, arginin, *r* and *l* prolin, histidin, lysin, tyrosin, leucin, isoleucin, and valin were isolated, while tryptophane was demonstrated by reaction. On the other hand, no positive sulphur test could be obtained, and mycol was found to be absent.

More recently, Robinson and Rettger (1917, 1918) have reported upon the growth and toxin production of *Bact. diphtheriae* when cultivated in a medium containing the product "opsine" previously studied by Dalimier and Lancereaux (1913). According to their description, "opsine is a biuret-free product resulting from the combined action of trypsin, erepsin and pepsin on certain protein materials not named by the authors or manufacturers." It was found to be abiuretic, and to give a high formol titration for monamino acids, the latter including leucine, tyrosin and very little tryptophane. In addition to the opsine, Robinson and Rettger attempted the cultivation of *Bact. diphtheriae* in media composed of the protein-free acid hydrolysis products of casein, edestin, and lactalbumin.

Notwithstanding rapid and abundant growth of the organism, they found that very little diphtheria toxin was formed in the opsine, the minimum lethal dose being 0.2 per cent of the body weight. In the acid hydrolysis products media, the growth was

considerably slower and less luxuriant than in the opsine and there was almost no evidence of any toxin. Addition of fresh beef infusion to opsine caused even more profuse growth of the bacilli than in diphtheria bouillon, while the toxicity was increased twenty-fold over that of cultures in opsine only. These authors consider that the results of their experiments tend to disprove the theory advanced by Uschinsky (1897) and also by Hadley (1907) that diphtheria toxin can be directly synthesized from comparatively simple nitrogenous substances like the amino acids. On the contrary, they support the view "that more complex bodies, perhaps some of the proteoses, as claimed by Hida (1908), or polypeptids, are essential to the formation of toxin." What little toxin is found in protein-free media, according to these investigators, may be formed from the disintegration products of dead bacilli during the long period of incubation.

It will be readily conceded from the above resume that comparatively little information is available regarding the constitution of diphtheria toxin. The two experimenters, Hadley and Uschinsky, who claim bacterial synthesis of the toxin, injected cultures of *Bact. diphtheriae* grown in their non-protein media for determination of toxicogenicity. This technique is, at once, open to the criticism that, with diphtheria bacilli, pathogenicity toward susceptible animals is not necessarily an indication of toxicogenicity. It would seem more likely that the death of the test animals was produced by the living pathogenic organisms which survived the long incubation periods rather than by the disintegration products of dead bacilli as has been suggested by Robinson and Rettger.

Observations made by one of us, Davis (1917), in connection with a study of bacteriologic peptone, showed that, when incorporated in bouillon, the presence or absence of certain amino acids in the peptone had a decided influence on the growth of *Bact. diphtheriae* and the production of its toxin.

The purpose of the work involved in the present paper was to determine, if possible, by cultivation of diphtheria bacilli in media consisting principally of known amino acids, the rôle played by these important protein constituents in the nutritional and toxicogenic reguirements of this organism.

EXPERIMENTAL PROTOCOLS

a. Preparation of materials

With the exception of sodium asparaginate, which was obtainable in sufficient purity, the amino acids and nitrogenous bases employed in the experimental media later described were individually prepared and their identity established. These bodies comprehended glycocoll, cystin, glutaminic acid hydrochloride, leucin, tyrosin, tryptophane, histidindichloride, glucoseamine hydrochloride, the purine bodies,—hypoxanthin and xanthin and the extractives,—creatin and creatinin.

Glycocoll was synthetically prepared, by the method of Nencki (1883), using powdered ammonium carbonate heated with monochloracetic acid. The glycocoll was isolated as the copper salt, decomposed with hydrogen sulphide, clarified with purified animal charcoal and crystallized. Several recrystallizations gave pure needles, answering all requirements for glycin.

Washed wool was found to be a very satisfactory raw material for cystin. The procedure given by Folin (1910) was followed with some modifications. The wool was hydrolized with hydrochloric acid, an excess of sodium acetate added, the precipitate washed, and then redissolved in boiling 5 per cent hydrochloric acid. After clarification with purified bone black, an excess of ammonium hydroxide was added, the resultant phosphate precipitate removed, and the material precipitated by addition of acetic acid. Thorough washing and drying gave typical hexagonal plates of pure cystin.

Glutaminic acid was prepared as the hydrochloride from both gliadin and glutenin of wheat flour, according to the directions given by Osborne and Guest (1911). Hydrolysis was carried out with concentrated hydrochloric acid, the material decolorized, using in this case repurified blood charcoal, and the colorless filtrate finally concentrated to syrupy consistency in vacuum. On cooling for several days, a heavy crystalline deposit was obtained which was repurified from alcohol. The resultant product left no ash on ignition and showed all characteristics of glutaminic acid hydrochloride.

Both leucin and tyrosin were obtained from blood clots using the separation method of Habermann and Ehrenfeld (1902). The clots were hydrolyzed with sulphuric acid, (5 : 13) made alkaline with milk of lime, and the lime removed as calcium oxalate. The filtrate was then concentrated to obtain a maximum yield of crude crystals, and the leucin was separated from the tyrosin by boiling the crystals in a mixture of glacial acetic acid and alcohol. After hot filtration, the tyrosin was clarified with bone black and recrystallized several times from cold water. Typical needles of tyrosin, giving a very strong Millon's reaction, were obtained. Additional quantities of this amino acid were also derived from the preparation of tryptophane, as will be subsequently described.

The hot alcohol-acetic acid filtrate containing the leucin was treated with an excess of copper carbonate, boiled, filtered, and the dark blue filtrate concentrated to crystallization. The crystals were decomposed with hydrogen sulphide, the liquid decolorized with bone black and then again evaporated to the appearance of crystals. A number of recrystallizations from water furnished silver-like plates of pure leucin.

A modification of the method of Hopkins and Cole (1902) was employed for the preparation of tryptophane, using Bacteriologic Peptone, (Parke, Davis and Company) as the source of raw material, which separated quantities of nearly pure tyrosin, requiring only crystallization to be suitable for use. The filtrate, after separation of tyrosin, was made 5 per cent acid with sulphuric acid and precipitation was effected with the mercuric sulphate reagent.

After thorough washing with sulphuric acid (5 per cent), the precipitate was decomposed with hydrogen sulphide and the latter removed with carbon dioxide. A partial precipitation with the mercuric sulphate reagent to remove cystin was now made, the precipitate removed, and an excess of the reagent added to the filtrate. The precipitate was again washed, decomposed with hydrogen sulphide, the latter removed with carbon dioxide, and whatever sulphuric acid was present was quantitatively removed with the requisite amount of barium

hydroxide. Filtration gave a clear liquid, which was mixed with half its volume of 90 per cent alcohol, concentrated, decolorized with purified blood charcoal, and again concentrated in vacuum. After several crystallizations from alcohol, rhombic crystals of tryptophane were obtained giving an intense reaction with Benedict's (1909) modification of the glyoxilic acid reagent.

Blood clots were also found to be the most satisfactory source for the preparation of histidin. Essentially, the procedure given by Pauly (1904) was followed, the clots being hydrolyzed with concentrated hydrochloric acid. The hydrolysate was then concentrated, made only weakly acid with sodium carbonate, filtered, and then made distinctly alkaline with the soda. After freeing from ammonia and diluting, mercuric chloride was added to produce precipitation. The precipitate was thoroughly washed, dissolved in hydrochloric acid (1:4) made alkaline with soda, and diluted, which caused the whole of the precipitate to be again thrown down.

Decomposition of the precipitate, after washing, was effected by hydrogen sulphide, the liquid was clarified with bone black, filtered, and the filtrate evaporated to syrupy consistency. Equal volume of absolute alcohol was now added, and the material concentrated in vacuum. On cooling and recrystallizing small rhomboid crystals were obtained, giving an intense color with Pauly's (1904) reagent and answering all requirements for histidin dichloride.

The hydrochloride of glucosamine was prepared by hydrolysis of chitin, obtained from decalcified lobster shells. Hydrochloric acid (1:2) was employed as the hydrolyzing agent, the liquid was clarified with blood charcoal and the filtrate evaporated to the appearance of crystals. The latter were recrystallized several times from water and finally were obtained pure in the form of parallelograms. In aqueous solutions, they strongly reduce Fehling's solution, have a distinct acid reaction to litmus and, on boiling with sodium hydroxde, are decomposed with elimination of ammonia.

Beef extract was used as raw material for hypoxanthin, xanthin and creatin, following the method given in Hawk's Practical

Physiological Chemistry (1916). Neutral lead acetate was added to precipitate the inorganic constituents, the excess of lead was removed with hydrogen sulphide, and the clear filtrate was concentrated to a thick syrup. After standing in the refrigerator for forty-eight hours, the syrup was extracted with 88 per cent alcohol which left a residue of crystals. The latter were dissolved in water, decolorized with bone black, the filtrate concentrated to small volume and allowed to crystallize. Repeated crystallization from water gave a substance which was identified as pure creatin. Creatinin was prepared from the creatin by hydrolyzing with dilute sulphuric acid. The excess of the latter was removed with barium carbonate, the material was filtered and evaporated to dryness. Extraction of the residue with 95 per cent alcohol and evaporation of the solvent gave pure creatinin.

The alcoholic filtrate from the above creatin preparation was evaporated to remove the alcohol, made ammoniacal, and precipitation effected by ammoniacal silver nitrate. The precipitate was now treated with boiling nitric acid (specific gravity of 1.1) filtered, and the hypoxanthine silver nitrate (identified by its crystalline appearance) was allowed to separate. The latter was decomposed by hydrogen sulphide, filtered, concentrated to remove the hydrogen sulphide, and make alkaline with ammonia. Free ammonia was removed by heating, the precipitate filtered, and the filtrate concentrated to small volume. On standing for several days in the refrigerator, small colorless needles of hypoxanthine were obtained.

To the filtrate from the hypoxanthine silver nitrate, ammonia was added in excess, the resultant precipitate filtered off and then decomposed with hydrogen sulphide. The filtrate was evaporated to a syrup and allowed to crystallize. Several recrystallizations gave pure xanthin.

The final reaction of all of the media described in the succeeding paragraph was adjusted to come within the limits, $\overset{+}{C}H = 1.0 \times 10^{-8}$ to $\overset{+}{C}H = 7.0 \times 10^{-9}$. This range has been found by Davis (1918) in a previous study on diphtheria toxin, to give

very satisfactory results in the routine production of high potency toxin. It is important to note, in this connection that it was found necessary to use the colorimetric method for accurate values of the media reactions. Electrometric determination of the hydrogen ion concentration of adjusted media containing amino acids proved to be unreliable, due to a steady increase in hydrogen ion concentration. In a typical case, the value rose steadily from an initial $\overset{+}{C}H = 9.5 \times 10^{-9}$ to about $\overset{+}{C}H = 2.5 \times 10^{-7}$ at the end of ten minutes.

Clark and Lubs (1917), in their work on hydrogen ion concentration, mention that synthetic media present some special problems in reaction and suggest the possibility of decomposition on sterilization. From the fact that the same phenomenon occurred on adjusting an unheated amino acid medium, the heat action can, in this instance, be left out of consideration. It is very likely that the increase is due to some action of the hydrogen while bubbling through the synthetic medium.

In all of the experimental work discussed below, the organism employed was a toxicogenic strain of *Bact. diphtheriae* originally obtained from Dr. W. H. Park and known as "Park no. 8". When grown in regular plain bouillon, under proper conditions, this culture has repeatedly furnished a toxin of which one L+ dose is less than 0.15 cc. A twenty-four hour culture in this medium shows a heavy pellicle and deposit with the intermediate bouillon entirely clear.

Unless otherwise noted, the general technic for toxicogenicity tests consisted in cultivating the organism in the experimental medium, contained in large flasks for 14 days at 37°C. The purity of the culture was then checked, 0.4 per cent of purified cresols were added, the mixed material allowed to stand in the refrigerator for twenty-four hours, and then filtered through a Mandler filter. By this method a clear product was obtained for test in which any toxic effects due to bacterial protein were eliminated. The toxicity was determined by subcutaneous injection of 250 gram guinea-pigs in accordance with the usual procedure for valuation of the potency of diphtheria toxin.

b. Laboratory data

1. Synthetic media. For preliminary experimentation looking toward the utilization of amino acids for the growth and toxin production of *Bact. diphtheriae*, a number of synthetic media were prepared. The various ingredients comprising each preparation were dissolved in a stated volume of distilled water and heated in flowing steam for fifteen minutes. The requisite amount of 10 N NaOH was then added to give a final reaction in the completed product of $P_H^+ = 8.0$ to $P_H^+ = 8.2$. This was readily accomplished by cold titration against N/10 NaOH to a deep pink color with phenolphthalein, following the technic cited above (Davis) 1918.

The medium was now steamed again for fifteen minutes and the reaction checked colorimetrically (using phenolsulphonphthalein and standardized H_3BO_3 – KCl – NaOH solutions) as already discussed. After filtration and distribution as desired, the material was sterilized for twenty minutes at 115°C.

It was apparent, at once, that a *Bact. diphtheriae* culture accustomed to peptone bouillon could not be successfully cultivated directly in synthetic media without preliminary acclimatization. Accordingly, successive transfers were made from the bouillon into mixtures of bouillon and synthetic medium containing gradually increasing quantities of the synthetic media until finally what might be termed a "limiting concentration" of the synthetic medium was obtained. The various experimental media are discussed below in detail; and the general results are summarized in table 1.

Synthetic medium no. 1

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	0.30	Creatinin	0.10
Tyrosine	1.00	Sodium asparaginate	1.40
Leucine	3.00	Cystin	0.40
Glutaminicacidhydrochloride	1.60	Sodium chloride	4.00
Glycocoll	0.40	Dipotassium acid phosphate	3.00
Creatin	0.10		

Typical luxuriant growth was obtained in twenty-four hours with this medium in mixtures containing up to 96 per cent synthetic medium and 4 per cent bouillon. With concentrations of the synthetic medium greater than 96 per cent, there was a marked decrease in the luxuriancy of the growth and pellicle formation. Above 98 per cent of synthetic medium, practically no growth was discernible. Notwithstanding the heavy growth at 96 per cent, toxicogenicity tests made by the regular procedure at this concentration showed practically no toxin. Guinea-pigs receiving doses of 0.1 cc. showed only irritation at the point of injection.

Synthetic medium no. 2.

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	0.40	Sodium asparaginate	1.20
Tyrosin	2.50	Histidindichloride	0.30
Leucin	2.50	Sodium chloride	4.00
Glutaminicacidhydrochloride	1.90	Dipotassium hydrogen phosphate	3.00
Glycocoll	0.85	Magnesium sulphate	0.50
Creatin	0.10	Potassium nitrate	0.20
Creatinin	0.10		
Cystin	0.40		

Very satisfactory growth, but practically no toxin was obtained in mixtures containing 98 per cent of the above medium and 2 per cent of plain bouillon. With greater proportions of the synthetic medium the same effects were noted as with medium no. 1. Precipitation of the tyrosin in the medium under consideration, showed an excess of this constituent. The following preparation was accordingly devised to contain a diminished quantity of this amino acid and also as a new factor, glucosaminehydrochloride, to note any increased action through carbohydrate addition.

Synthetic medium no. 3

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	0.60	Histidindichloride	0.50
Tyrosin	0.80	Glucosaminehydrochloride	1.50
Leucin	3.00	Sodium asparaginate	0.50
Glutaminicacidhydrochloride	2.50	Sodium chloride	2.50
Cystin	0.40	Dipotassium acid phosphate	3.00
Creatin	0.20	Magnesium sulphate	0.40
Glycocoll	0.80	Potassium nitrate	0.20

Practically the same results as regards growth and toxicogenicity were obtained with this preparation as with the preceding medium no. 2. The protocols of the foregoing experiments indicated that, in all probability, the preparation contained a number of superfluous constituents. Medium no. 4 was accordingly made up with only three amino acids and glucosaminehydrochloride, but with increased amounts of each of these substances.

Synthetic medium no. 4

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	1.25	Sodium chloride	2.50
Histidindichloride	1.25	Dipotassium acid phosphate	3.00
Cystin	0.42	Magnesium sulphate	0.40
Glucosaminehydrochloride	2.75		

Heavy typical growth was obtained in this case with 95 per cent of synthetic medium as the "limiting" concentration. With a greater proportion of synthetic medium than 97 per cent, almost no growth was obtained. As was expected, the toxicogenicity results were negative.

Synthetic medium no. 5

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	0.60	Creatinin	0.08
Tyrosin	1.25	Xanthin	0.05
Leucin	3.00	Hypoxanthin	0.05
Glutaminicacidhydrochloride	2.50	Sodium chloride	4.00
Glycocoll	0.85	Dipotassium acid phosphate	3.00
Sodium asparaginate	1.00	Magnesium sulphate	0.50
Cystin	0.40	Glucose	1.50
Histidindichloride	0.50	Potassium nitrate	0.20
Creatin	0.20		

With this preparation, which was the most comprehensive synthetic medium investigated, heavy, typical growth was obtained in a mixture containing as high as 99.5 per cent of the synthetic and only 0.5 per cent of plain bouillon. A slight growth with slight pellicle formation was obtained even with 99.8 per cent of synthetic medium and only 0.2 per cent of plain bouillon, but with a concentration of bouillon less than the latter amount, the growth failed.

It was deemed of interest, in connection with this medium, to determine what proportions of the synthetic medium and bouillon gave maximum toxin production. It was found that a toxin having an L+ dose of 0.15 cc. was still obtained where the mixture contained 90 per cent synthetic medium and 10 per cent bouillon. At the "limiting" concentration of 99.5 per cent, however, even with an apparently maximum growth, potency valuations showed practically no elaboration of toxin, which corroborates previous findings with synthetic media.

By way of possibly obtaining some information as to the important constituents in the above medium, a number of mixtures were prepared containing 99 per cent of differing synthetic media together with 1 per cent of plain bouillon. Inoculation of each was made directly from cultures grown in a 99 per cent mixture of medium no. 5. The composition of the preparations is given below:

Synthetic medium no. 6

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	1.60	Sodium chloride	2.50
Cystin	0.40	Dipotassium acid phosphate	3.00
Glutaminicacidhydrochloride	2.50	Magnesium sulphate	0.40
Glucosaminehydrochloride	1.50		

The transfers into a 99 per cent mixture of the above showed only a scant growth, indicating this to be an inferior medium to no. 5 for cultivating *Bact. diphtheriae*. On addition of sufficient bouillon to reduce the concentration of synthetic medium to 97 per cent, maximum intensity of growth was again restored.

Synthetic medium no. 7

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	1.00	Dipotassium acid phosphate	3.00
Cystin	0.75	Magnesium sulphate	0.40
Sodium chloride	2.50		

Almost no growth was obtained here in a 99 per cent mixture, and only very scant growth with the bouillon concentration raised to 5 per cent. A reduction of the synthetic medium concentration to 90 per cent was found to be necessary before luxuriant, typical growth could be obtained.

Synthetic medium no. 8

CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	1.60	Histidindichloride	1.20
Cystin	0.40	Sodium chloride	2.50
Glutaminicacidhydrochloride	2.50	Dipotassium acid phosphate	3.00
Glucosaminehydrochloride	1.00	Magnesium sulphate	0.40

Heavy typical growth, comparable in character and intensity to that obtained in dilutions of medium no. 5, was obtained in 99 per cent mixtures of the above preparation. The luxuriance in this case, however, began to diminish at 99.2 per cent and no growth was obtained in a mixture of 99.5 synthetic medium and 0.5 plain bouillon.

Medium no. 9. The fact that it was impossible to maintain metabolism of the microorganism in question on any of the preceding synthetic media alone, suggested that some important constituent might be lacking. By way of attacking the problem from a different angle, gelatine was hydrolyzed with 25 per cent sulphuric acid for twenty-four hours on the sand bath. Sufficient barium hydroxide was now added to make the hydrolysate distinctly alkaline and the resultant heavy precipitate was filtered off. The excess of barium hydroxide was next exactly neutralized with 10 per cent sulphuric acid, the precipitate removed and the clear filtrate (tested to prove absence of both Ba and SO_4 ions) was concentrated in vacuum to a thick syrup.

From the work of Levene and Beatty (1906), it appears that gelatin is lacking in cystin, tyrosine and tryptophane. To supply this deficiency in the above hydrolysate, the syrup was diluted with distilled water to a total solids concentration of 2 per cent and the following were added to each liter of medium:

CONSTITUENTS	AMOUNTS OF 1000 CC. OF DISTILLED WATER	CONSTITUENTS	AMOUNTS IN 1000 CC. OF DISTILLED WATER
	grams		*grams*
Tryptophane	0.40	Sodium chloride	5.00
Tyrosine	1.50	Dipotassium acid phosphate	3.00
Cystin	0.40	Magnesium sulphate	0.50

The resultant product was prepared, distributed, and sterilized as already described, and acclimatization studies were carried out as with the straight, synthetic media. Contrary to expectations, both the macroscopical appearance and the morphological characteristics of the organisms under investigation indicated that this medium was inferior to most of the preceding

preparations. A mixture containing 90 per cent of the synthetic medium and 10 per cent of plain bouillon appears to be the "limiting" concentration permitting maximum growth and pellicle formation. Even at this dilution of the medium, the growth is not as vigorous and the formation of pellicle appears more scanty than obtained in the unmodified preparations. Corroborating the foregoing, toxicogenicity results were negative.

TABLE 1

Growth and toxicogenicity of Bact. diphtheriae in synthetic media

MEDIUM NUMBER	MAXIMUM GROWTH CONCENTRATION		TOXICOGENICITY RESULTS	REMARKS
	Synthetic	Bouillon		
	per cent	*per cent*		
1	96.0	4.0	Negative	Organisms shorter, stain solid
2	98.0	2.0	Negative	Appearance more typical
3	98.0	2.0	Negative	Same appearance as no. 2
4	95.0	5.0	Negative	Growth not as heavy as preceding
5	99.5	0.5	Negative	Toxin having L+ dose = 0.15 cc obtained with 90 per cent synthetic, 10 per cent bouillon
6	97.0	3.0	Negative	Heavy growth light pellicle formation
7	90.0	10.0	Negative	Short, plump bacilli, stain solid
8	99.0	1.0	Negative	Heavy, typical growth and pellicle formation
9	92.0	8.0	Negative	Inferior growth scanty pellicle
10	90.0	10.0	Negative	Same as preceding

Medium no. 10. The fact that gelatine is of animal origin suggested the use for a culture medium of a vegetable protein hydrolysate. For this purpose, gliadin, the prolamine obtained from wheat, was prepared, according to the procedure given by Osborne and Clapp (1907). These investigators have shown that lysine is absent in gliadin, and that this protein contains only 0.02 per cent of glycocoll.

The gliadin was hydrolyzed exactly as carried out with the gelatin, using both the 25 per cent sulphuric acid and the barium hydroxide, and the final product, containing neither Ba nor SO_4 ions, was also diluted to a final total solids content of 2 per cent. Equal parts of this hydrolyzed product and of the fin-

ished medium no. 9 were then taken, 0.2 gram of tryptophane added per liter, the reaction adjusted, and this composite was then finished in the usual way. As will be noted from Table 1, practically the same results were obtained with this experimental preparation as with medium no. 9, and here, also, toxin production was unsuccessful.

2. Elimination media. It appears to be clearly indicated from the preceding experiments that metabolism of *Bact. diphtheriae* is not permitted in a medium composed principally of amino acids. That the amino acids are, however, important factors in both growth and toxin elaboration with this organism seems to be plainly evidenced from the results already detailed with medium no. 5. The latter have also demonstrated that not all of these protein degradation products can exercise the same influence.

Preliminary cultivation in plain beef infusion carried out with the *Bact. diphtheriae* strain employed above, showed that only a scant growth with no formation of toxin is possible in this medium. A Liebig's Extract of Beef solution gave even poorer results. More luxuriant growth but still no toxicogenicity occurred in a 2 per cent solution of peptone.

With the preceding facts in mind, several series, of what might be termed "elimination" media, were now devised having in each series one or two of the above three substances as basic ingredients. The individual amino acids or the other materials incorporated in the synthetic media already investigated were then added in the same quantities as previously employed. Adjustment of the reaction and finishing of these media were in accordance with the usual technic. In order to acclimatize the diphtheria culture to the various media, the organisms in each case were cultivated first for three successive generations in "starter" flasks containing 30 cc. of the special broths and then inoculated into the large flasks.

The experimental preparations, together with the results obtained, are detailed in the accompanying table 2. As may be noted from the table, five series of the "elimination" media were prepared. The first set contained plain beef infusion as the

basic ingredient, the second had 2 per cent peptone (Bacteriologic, Parke, Davis and Company) solution, and for the third series, a 1 per cent Liebig's Extract of Beef solution served as the common constituent. In the remaining two sets, ordinary bouillon (2 per cent peptone, beef infusion and 0.5 per cent of sodium chloride) and a meat extract bouillon (2 per cent peptone, 0.5 per cent salt, 1 per cent Liebig's Extract) were used as basic constituents.

The presence of any of these basic media in a preparation is indicated in the table by a (*) and the absence of the others, by the symbol (−). The intensity of the growth has been characterized as follows: (++++) = heavy; (+++) = good; (++) = moderate, and (+) = scant. In the valuation of toxin potency, it was not deemed necessary to determine the minimum lethal dose (M. L. D.) above 1 cc., but where such results were obtained they have been designated by "M. L. D. = 1 cc. +."

Probably the most significant of the data presented in table 2 are the results furnished with beef infusion alone, as a basic medium. Where 2 per cent peptone solution served as the common ingredient, practically no differences could be distinguished on addition of the special constituents. In each case, only the moderate growth due to peptone itself was obtained. Except where cystin was added, the results given with Liebig's Extract were negative.

The purpose in using regular (peptone beef infusion) bouillon and Liebig's Extract (peptone) bouillon as basic media was to determine whether addition of the amino acids and other special constituents caused any increase in potency of the toxin. As may be noted from table 2, the final toxicities furnished by the plain bouillon, special combinations are no higher than those given by the same culture of *Bact. diphtheriae* when cultivated in the plain bouillon alone. Confirming expectations, the special media containing Liebig's Extract gave results uniformly inferior to beef infusion and, in no case, a final toxin of which, one L+ dose was less than 1 cc.

TABLE 2

Effect of special constituents on growth and toxicogenicity of Bact. diphtheriae

CONCENTRATION OF SPECIAL CONSTITUENTS IN 1000 cc.	BASIC MEDIA: Beef infusion	BASIC MEDIA: 2 per cent peptone	BASIC MEDIA: 1 per cent Liebig's extract	CHARACTER OF GROWTH	POTENCY OF TOXIN	REMARKS
Tryptophane, 0.60 gram	*	–	–	++++	M. L. D. = 0.01 cc.	Strong growth with beef infusion base. L + dose of toxin = 0.75 cc.
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Cystin, 0.50 gram	*	–	–	++++	M. L. D. = 0.0075	L + dose of toxin with been infufusion = 0.5 cc.
	–	*	–	++	M. L. D. = 1 cc.	
	–	–	*	+++	M. L. D. = 1 cc.	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Tyrosin, 1.25 grams	*	–	–	++	M. L. D. = 1 cc. +	Only moderate growth with beef infusion base
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Glutaminic acid hydrochloride, 2.50 grams	*	–	–	++++	M. L. D. = 0.05 cc.	Growth with beef infusion not as heavy as with cystin
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Histidin dichloride, 0.50 gram	*	–	–	+++	M. L. D. = 1 cc. +	Growth with beef infusion base comparable to tyrosine
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	++	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Leucin, 3.00 grams	*	–	–	++	M. L. D. = 1 cc. +	Moderate growth only with beef infusion base
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Glycocoll, 0.75 grams	*	–	–	++++	M. L. D. = 0.07 cc.	Beef infusion growth not as heavy as with glutacid HCl
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	

TABLE 2—*Continued*

CONCENTRATION OF SPECIAL CONSTITUENTS IN 1000 cc.	BASIC MEDIA: Beef infusion	BASIC MEDIA: 2 per cent peptone	BASIC MEDIA: 1 per cent Liebig's extract	CHARACTER OF GROWTH	POTENCY OF TOXIN	REMARKS
Sodium asparaginate 1.5 grams	*	–	–	++++	M. L. D. = 0.05 cc.	Behavior same as with glutaminic acid hydrochloride
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Creatin, 0.2 gram; creatinin, 0.15 gram	*	–	–	+++	M. L. D. = 1 cc. +	Scant growth with beef infusion base
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Xanthin, 0.05 gram; hypoxanthin, 0.05 gram	*	–	–	+	M. L. D. = 1 cc. +	Beef infusion growth same as preceding
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
Glucose	*	–	–	+	M. L. D. = 1 cc. +	Beef infusion growth same as with creatin
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.25 cc.	
	–	*	*	+++	L + = 1.5 cc.	
Glucosamine HCl, 2.00 grams	*	–	–	+	M. L. D. = 1 cc. +	Behavior same as with glucose
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.2 cc.	
	–	*	*	+++	L + = 1.5 cc.	
Control	*	–	–	+	M. L. D. = 1 cc. +	Behavior same as preceding
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	
NaCl, 4 grams; K_2HPO_4, 3 grams $MgSO_4$, 0.4 grams; KNO_3, 0.2 grams	*	–	–	+	M. L. D. = 1 cc. +	Behavior same as preceding
	–	*	–	++	M. L. D. = 1 cc. +	
	–	–	*	+	M. L. D. = 1 cc. +	
	*	*	–	++++	L + = 0.15 cc.	
	–	*	*	+++	L + = 1 cc.	

The addition of cystin to plain beef infusion as may be seen from table 2 not only permits heavy typical growth with *Bact. diphtheriae*, but a relatively strong toxin is also elaborated. Tryptophane, similarly, allows a heavy growth, but the resultant toxin is not as active as obtained with cystin. Practically the same results were obtained on adding either glutaminic acid hydrochloride or sodium asparaginate to the beef infusion. *Bact. diphtheriae* grew luxuriantly in both cases, yielding a final toxin, however, which had only one-fifth the strength of that obtained with tryptophane. The growth in presence of glycocoll, it will be noted, was not as heavy as with the two preceding constituents, and the filtered toxin was also weaker (minimum lethal dose equals 0.07 cc.).

Tyrosin, leucin, and histidindichloride, when added to beef infusion, each appear to yield the same results. They permit a moderate growth of the organism, but no toxin of consequence is elaborated. Comparison of the protocols given by the remainder of the special constituents investigated, with those furnished by the control preparations shows no essential difference. These include the results obtained with creatin and creatinin, xanthin and hypoxanthin, the hydrochloride of glucosamine, glucose, the inorganic ingredients, sodium chloride, magnesium sulphate, potassium nitrate and dipotassium hydrogen phosphate. The growth of the bacillus and formation of toxin in the various "control" media have already been discussed.

DISCUSSION

Notwithstanding the fact that the amino acids by themselves appear to be unable to support the metabolism of *Bact. diphtheriae*, some of these protein degradation products, as has been shown in the foregoing, have a decided influence on growth and toxin production with this organism. Of the various amino acids investigated, cystin seems to be of special importance. Addition of this sulphur containing body not only permits heavy typical growth of the diphtheria bacillus in a medium like beef infusion which is merely capable of maintaining metabolism of *Bact. diphtheriae*, but simultaneously the organism is now able to

elaborate a potent toxin. That this toxin is a true diphtheria toxin is amply demonstrated by its ability to neutralize diphtheria antitoxin giving an L+ value for potency closely corresponding with the theoretical value derived from determination of the minimum lethal dose.

The fact that tryptophane in beef infusion allows a growth of *Bact. diphtheriae* practically of the same intensity as does cystin, while the resultant toxin is of inferior potency, would suggest that the sulphur complex in the cystin molecule enters into toxin production. It seems likely that diphtheria toxin contains sulphur and that where tryptophane and other non sulphur containing amino acids permit toxin elaboration, with beef infusion, the sulphur portion of the toxin molecule is derived from the infusion. Indol tests made after growth of the organism in tryptophane containing media were found to be negative, indicating that in the utilization of this compound by the diphtheria bacillus, there is no intermediate separation of the indol ring.

The very close relationship of asparaginic acid (amino succinic acid) to its next higher homologue, glutaminic acid (amino glutaric acid) appears to be closely paralleled by the similarity in the protocols furnished by both glutaminic acid hydrochloride and sodium asparaginate. The growth of the organism is of equal intensity in presence of either of these substances and a toxin of practically the same potency is elaborated in both cases.

It is worthy of note that glycocoll added to beef infusion with a reaction adjusted to the proper H ion concentration permits of heavy growth and toxin elaboration with *Bact. diphtheriae*. Since glycocoll has no asymmetric carbon atom, it might be deduced that the major factor contributing to formation of toxin in this case need not necessarily be optically active. Furthermore, although equivalent results were obtained with both sodium asparaginate and glutaminic acid hydrochloride, the glutaminic acid is dextro rotatory while the asparaginic acid turns the plane of polarized light to the left. Seemingly, the direction of rotation is also of minor consequence.

The fact that it was found impossible to grow *Bact. diphtheriae* satisfactorily and obtain a production of toxin except in the

presence of beef infusion, even though the quantity of the latter was relatively very small, suggests a food hormone requirement for growth, and particularly for toxin production, by the organism. Davis (1917) has discussed the question of food accessory factors with reference to cultures of hemophilic bacilli and both Lloyd (1917) and Drew (1917) with the meningococcus. They all conclude that the presence of these factors is essential. Assuming that both peptone and Liebig's Extract of beef are deficient in vitamines, and that ordinary beef infusion has these factors present, this may partially explain the scant growth and absence of toxin which obtained in presence of both the peptone and the extract.

It is readily appreciated that the preceding experimentation has not directly included other important amino acids, particularly proline and lysine, although these have been comprehended in the hydrolysis media nos. 9 and 10 detailed in table 1. The experimental data obtained, however, warrant the belief that it is not possible to maintain metabolism of *Bact. diphtheriae* in a medium composed entirely of amino acids, even after addition of muscle extractives and mineral salts.[2] This lends strength to the theory that diphtheria toxin is an excretory product formed by the organism only when certain amino acids and accessory factors, the latter perhaps of a vitamine character, are present. It does not seem likely, as has been claimed by several, that the toxin is a synthetic product, built up directly by *Bact. diphtheriae* from mineral salts and nitrogen compounds of the character of amino acids.

SUMMARY

1. *Bact. diphtheriae* could not be cultivated in synthetic media composed of amino acids and mineral salts adjusted to the optimum H ion concentration. Addition of the extractives creatin and creatinin and the purine bases, xanthin and hypoxanthin, was of no advantage.

[2] Experimentation in progress since this paper was read indicates that growth of *Bact. diphtheriae* in a 100 per cent synthetic medium is possible. This will be discussed in a later publication.

2. Typical luxuriant growth of *Bact. diphtheriae* was obtained in a mixture of 99.5 per cent synthetic medium and only 0.5 per cent of bouillon. Production of active toxin, however, required the presence of 10 per cent bouillon.

3. Cystin, when added to plain beef infusion, a culture medium, just capable of maintaining growth of *Bact. diphtheriae*, not only permitted rapid and heavy vegetation, but a strong toxin (minimum lethal dose equals 0.0075 cc.) was also elaborated. This appears to be a true toxin capable of neutralizing the corresponding diphtheria antitoxin.

Tryptophane, under the same conditions, allowed almost as heavy growth, but the resultant toxin (minimum lethal dose equals 0.01 cc.) was weaker in potency. Glutaminic acid hydrochloride and sodium asparaginate gave parallel results. The growths were not as heavy as with cystin or tryptophane and, in both cases, the toxin produced had only one-fifth of the strength (minimum lethal dose equals 0.05 cc.). Heavy typical growth was also obtained with glycocoll, but the toxin elaborated (minimum lethal dose equals 0.07 cc.) was much less potent.

Moderate growth and practically no toxin production by *Bact. diphtheriae* were obtained in beef infusion containing leucin, tyrosin, or histidindichloride. Addition to beef infusion of any of the following: creatin and creatinin, xanthin and hypoxanthin, glucose, glucoseamine hydrochloride, and the inorganic salts, sodium chloride, dipotassium hydrogen phosphate, magnesium sulphate, and potassium nitrate permitted only scant growth and consequently no toxin. Substitution of the beef infusion by either peptone or Liebig's Extract resulted in deficient growth and toxin formation. This, and the data furnished by the synthetic media suggest a vitamine requirement not only for luxuriant growth of the organism but particularly for strong toxin production.

4. The results obtained favor the belief that diphtheria toxin is not a synthetic product, but rather a catabolic substance elaborated by *Bact. diphtheriae* only in presence of certain amino acids and accessory factors, the latter probably of a vitamine character.

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THE INTERPRETATION OF B. COLI TEST RESULTS ON A NUMERICAL AND COMPARATIVE BASIS

AS APPLIED TO THE INVESTIGATION OF WATER AND SEWAGE

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One of the principal shortcomings of the *B. coli* test lies in the interpretation of the results obtained. A statement of the number of colon bacilli present in the liquor tested is the only presentation of results which would be intelligible to the layman, and an analysis of professional discussions as to the interpretation of *B. coli* tests for specific purposes will often disclose that one of the things befogging the issue is the failure to agree on what a certain percentage of positive tests really means.

By use of the theory of probabilities, it is possible to interpret the results as usually obtained in terms of definite numerical values. The writer devised a method for doing this several years ago, of which a synopsis was published in the Engineering News-Record of May 25, 1917. Various queries and comments relative thereto have prompted the preparation of this article. For purposes of identification this method of interpreting *B. coli* results will be referred to as the *B. coli* factor method.

The principal objects of this article are:

1. To show the rationality of the *B. coli* factor method by developing the same from a few universally accepted elementary conceptions in probabilities.

2. To discuss the errors to which the *B. coli* test is subject and to show how these errors may be evaluated.

3. To present in the form of a chart the necessary data from which, given a certain percentage of positive results in a series of tests, the number of *B. coli* per cubic centimeter and the expected errors in the result can be obtained at once, without computation,

thereby making this method available to those with a limited mathematical training and without the consumption of the time required for computation by methods now in vogue.

4. To propose a means for handling the troublesome question of variation in *B. coli* content from test to test and its effect upon average values.

The author believes the method here given to be rationally sound, practicable, and easy of application. He wishes to give warning, however, that with this, and with any method that might be devised, the reliability of results depends upon a sufficiency of tests, and upon the alertness and good judgment of the analyst in accommodating his samples: in size, to the variable *B. coli* content of the water; and in time, to the fluctuations therein. This method offers means of assisting in the formation of judgment on these factors, and avoids the compounding of tests of different degrees of accuracy, which leads to confusing and irrational results.

MATHEMATICS OF THE B. COLI FACTOR

The basis of this method for expressing numerically the results of a series of *B. coli* tests is the mathematical theory of probabilities. Moreover, it can be developed directly from the fundamental conception of probability as used in that theory. This is often illustrated by the following example; if a bag contains nine white balls and one black ball and a ball is withdrawn at random, the probability that it is black is one-tenth. Stated as a general case: if of t equally likely occurrences, f are favorable to a certain event E, then the probability of its occurrence is given by the proper fraction $\frac{f}{t}$, or

$$P(E) = \frac{f}{t} = p$$

Since one of the t occurrences is certain to happen, the fraction representing the probability in this case being $\frac{t}{t}$ or 1, 1 is the

mathematical representation of certainty. It then follows that the probability of the non-occurrence of the event E is $1 - p$ or

$$P(\overline{E}) = 1 - p = q$$

This conception of the probability of an event is confirmed by logicians and philosophers, and is accepted by common usage, as in games of chance and in discussing the possible success or failure of an enterprise, when it is common to say that it has one chance in a certain number of succeeding, although the fraction so chosen is usually a guess, pure and simple. So that the starting point in the development of this method is a universally accepted axiom based largely on common experience.

The next step relates to a series of trials. If, instead of one trial, as above, s successive trials are made, with the understanding that the ratio of favorable cases, or p, does not change during the trials (which means in the white and black ball scheme, that the balls withdrawn are replaced after each trial or else that the total number is so large that the ratio is not appreciably affected by the withdrawals during the series of trials) then the probabilities of the favorable event occurring s, $s - 1$, $s - 2$, . . . 0 times are given by the values of the terms of the expansion of $(p + q)^s$ of which s, $s - 1$, $s - 2$, etc., are the exponents of p. While this is susceptible of exact proof, such proof would be too lengthy for this discussion. However, an example may serve to show the manner of its derivation. Suppose a bag containing 10 per cent black balls, the rest being white, and suppose four successive drawings to be made (with replacements). Then $p = 0.1, q = 0.9, s = 4$, and the probabilities of obtaining 4, 3, 2, 1 or 0 black balls are given by the terms of the expansion $(0.1 + 0.9)^4$ which by the binomial theorem are:

$$0.1^4 + 4 \times 0.1^3 \times 0.9 + 6 \times 0.1^2 \times 0.9^2 + 4 \times 0.1 \times 0.9^3 + 0.9^4$$

Evaluating these terms:

Probability of obtaining 4 black balls	= 0.0001
Probability of obtaining 3 black balls	= 0.0036
Probability of obtaining 2 black balls	= 0.0486
Probability of obtaining 1 black ball	= 0.2916
Probability of obtaining 0 black balls	= 0.6561
Total = Certainty	= 1.0000

In the first term, which gives the probability of drawing four black balls, it is evident that there are nine chances of drawing a white ball, and one chance of drawing a black ball at the first trial. Anyone of these ten balls having been drawn, anyone of ten other balls may be drawn on the second trial, giving ten possible combinations with the ball first drawn, or 100 possible combinations with the ten balls anyone of which may have been drawn on the first trial. Similarly any one of ten balls may be drawn at the third trial with each of the 100 possible combinations of the first two trials giving 1000 combinations, and at the fourth trial any one of ten with the 1000 combinations of the first three trials, giving a total of 10,000 possible combinations for the four trials. Of these, there is only one possible combination for drawing four black balls, or one chance in ten thousand giving by the definition:

$$P_4 = 1 \div 10{,}000 = 0.0001$$

In the second term, which gives the probability of drawing three black and one white ball, and in each of the others, there are evidently also 10,000 possible combinations of four balls to be obtained in the four trials. Assuming that it is desired to draw first three black balls and then a white ball in succession, there is one chance in a thousand of obtaining the one possible combination of three black balls in the total 1000 possible combinations of the first three trials. If this rare combination is obtained, there are evidently nine chances out of ten for obtaining a white ball at the fourth drawing, so that there are nine combinations of the possible 10,000 which give three black balls and one white ball in the desired sequence. If the order of drawing is immaterial, the following arrangements may be obtained:

	1	2	3	4
First drawing	●	●	●	○
Second drawing	●	●	○	●
Third drawing	●	○	●	●
Fourth drawing	○	●	●	●

thus giving a total probability of

$$p_3 = 4 \times 9 \div 10{,}000 = 0.0036$$

In the third term, which gives the probability of drawing two black and two white balls, there is one chance in 100 of drawing two black balls in the first two trials. Assuming this to have been accomplished there are nine chances out of ten of drawing a white ball at the third trial, and with each of these nine there are nine additional chances of drawing a white ball at the fourth trial giving a total of $1 \times 9 \times 9$ or 81 chances in the 10,000 possible ones of attaining the desired result in the sequence given. The possible sequences of the two white and two black balls are:

	1	2	3	4	5	6
First drawing	●	○	○	●	○	●
Second drawing	●	●	○	○	●	○
Third drawing	○	●	●	●	○	○
Fourth drawing	○	○	●	○	●	●

thus giving a total probability of

$$p_2 = 6 \times 81 \div 10{,}000 = 0.0486$$

In the fourth term, which gives the probability of drawing one black and three white balls, there is one chance in ten of drawing a black ball at the first trial, nine chances in ten of drawing a white ball at the second trial, at the third trial and at the fourth trial, and there are four possible orders in which one black and three white balls may be drawn in the four trials, so that the reasoning is the same as in the second term, with p and q reversed, however.

The fifth term is derived by reasoning analogous with that used in the first term.

This rather lengthy example shows that there are no abstruse manipulations or dubious assumptions in finding the probabilities in a series of trials, but that this involves merely finding the number of combinations favorable to an event and

the total number of possible combinations and then applying the fundamental definition of probability as a ratio of the two.

Now, in the *B. coli* test, a definite quantity of the liquid to be examined is placed in a fermentation tube containing media from which the colon bacilli, if present can produce gas, which being trapped in the closed end of the tube, indicates their presence. If we ascertain the volume of such a bacillus, and divide a comparatively large volume of the sample by this volume we can obtain the number of droplets of liquid equivalent in size to the colon bacillus. Assuming that we know by some means, the number of colon bacilli in the sample, we can consider these as black balls, and the water droplets of equal size as white balls. Then the ratio of the number of coli to the total number of droplets gives the value of p or the probability of obtaining a colon bacillus by withdrawing one droplet from the sample.

Let us suppose that the amount used in the test (0.1, 1, 10 cc.) contains s droplets, then the probabilities of obtaining s, $s - 1$, $s - 2$, etc., coli would be equal to the terms of the expansion $(p + q)^s$ as already explained above. However, we only know from the presence or absence of gas in the test, whether one or more *B. coli* are present, or whether they are entirely absent. The question then is, how, from a number of tests, some positive and some negative, we can deduce the most probable number of *B. coli* per cubic centimeter. The last term of the expansion $-q^s$ — gives the probability of withdrawing no *B. coli* with the quantity taken for test, therefore $1 - q^s$ must give the total probability of obtaining one or more *B. coli* in the quantity tested, or the probability of obtaining a gas indication in the test. From one test no deduction can be drawn, but in a number of tests, the ratio of the positive results to the total number will approximate this probability of $1 - q^s$, which being designated by P, we may write the following:

$$\operatorname*{Lim}_{N = \infty} P = 1 - q^s \qquad \text{(I)}$$

Where N is the number of tests made.

The greater the number of tests, the more closely will the ratio of positive to total tests approach this value of P, the accuracy increasing as the square root of the number of tests.

The use of equation I is tedious due to the necessity of reducing the sample to droplets the size of bacteria (of which there would be about 500,000,000,000 per cubic centimeter) resulting in numbers too large to handle, besides involving an element of doubt as to the actual size of the bacteria. Larger particles would serve equally well, distinguishing between those containing *B. coli* and those not containing any. If taken too large, the results obtained would be incorrect, since this would reduce the number of particles too much, as the theory of probabilities assumes the use of large numbers. This difficulty can however be entirely surmounted and all doubt as to the size of particles and uncertainty of results caused thereby eliminated as follows: Let

Q = the proportion of negative tests obtained, so that

$$Q = 1 - P$$

m = the number of units containing one *B. coli*, so that

$$\frac{1}{m} = p; \quad 1 - \frac{1}{m} = q$$

xm = number of units tested (corresponding to s as used above) x being any whole number or fraction, and representing the number of coli in the sample tested.

Then

$$Q = q^x = \left(1 - \frac{1}{m}\right)^{xm} \qquad \text{(II)}$$

Letting $x = 1$, $Q = \left(1 - \frac{1}{m}\right)^m$

Expanding this by the binomial theorem:

$$\left(1 - \frac{1}{m}\right)^m = 1^m - m \cdot 1^{m-1} \cdot \frac{1}{m} + \frac{m\,(m-1)}{2} \cdot 1^{m-2} \cdot \frac{1}{m}2 - \frac{m\,(m-1)\,(m-2)}{2\,.\,3} \cdot 1^{m-3} \cdot \frac{1}{m}3, \text{ etc.} \qquad \text{(A)}$$

The first two terms, each equal to 1, cancel, the remaining terms being multiplied out, and reduced to a common denominator give, approximately:

$$\left(1-\frac{1}{m}\right)^m = .3678 \text{ etc.} - \frac{1}{6m} - \frac{1}{6m^2} + \frac{1}{6m^3} - \frac{1}{5m^4}, \text{ etc.} \quad \text{(B)}$$

By making m sufficiently large, all the terms following the first may be made as small as desired, and so can be neglected.

It can be shown that the exact value of the first term of this modified series is, when evaluating the first $n+2$ terms of series A:

$$\text{First term} = \frac{1}{2!} - \frac{1}{3!} + \frac{1}{4!} - \frac{1}{5!}. + \frac{1}{6!} - \frac{1}{7!} \cdots \pm \frac{1}{(n+1)!}*$$

Evaluating the first eight terms of this series gives

$$\operatorname{Lim}_{m=\infty}\left(1-\frac{1}{m}\right)^m = 0.367879 \text{ (correct to 5 places)}$$

*The $(n+2)$ 'd term of series A contains $n+1$ factors in the numerator, each involving m, which by multiplication give an expression the first term of which is m^{n+1}. The denominator of the $)n+2)$'d term is $(n+1)!\, m^{n+1}$. So that this term may be written

$$\frac{m^{n+1} + am^n + bm^{n-1} + cm^{n-2} \cdots, \text{ etc.}}{(n+1)!\ \ m^{n+1}}$$

or

$$\frac{1}{(n+1)!} + \frac{a}{(n+1)!\,m} + \frac{b}{(n+1)!\,m^2} + \frac{c}{(n+1)!\,m^3} + \cdots, \text{ etc.}$$

The $(n+1)$ 'st term, similarly may be written

$$\frac{m^n + gm^{n-1} + hm^{n-2} + im^{n-3} + \cdots, \text{ etc.}}{n! \cdot m^n}$$

or

$$\frac{1}{n!} + \frac{g}{n!\,m} + \frac{h}{n!\,m^2} + \frac{i}{n!\,m^3} + \cdots, \text{ etc.}$$

and so on for the other terms, a, b, c—g, h, i—are unknown constants, which may be positive or negative.

Summing up all the terms in which the variable m is absent as the first term of equation B, those containing $\frac{1}{m}$ as the second term, those containing $\frac{1}{m^2}$ as the third term, etc., it is evident that the first term takes the form given.

his value is the reciprocal of the Naperian Base e or we may write

$$\lim_{m=\infty} \left(1 - \frac{1}{m}\right)^m = \frac{1}{e}$$

Substituting in formula (II), this gives:

$$Q = \left(\frac{1}{e}\right)^x = e^{-x} \qquad \text{(III)}$$

Whence $\log Q = -x \cdot \log e$, or using the base e

$$-x = \log_e Q; = \log_e (1 - P) \qquad \text{(IV)}$$

The significance of the minus sign is simply that since Q is a proper fraction, its logarithm is negative, thereby cancelling the minus sign before the x.

Since x is the number of *B. coli* in the sample tested, by using 1 cc. samples in our computations, we can obtain the *B. coli factors* for various percentages (P) of positive tests, so that it will only be necessary to divide the proper *factor* by the size of the sample used to obtain the number of *B. coli* per cubic centimeter. These factors have been computed and plotted in figure 1.

EXPECTED ERRORS[1]

The results obtained by the method heretofore developed are subject to errors of sampling usual to values based upon obser-

[1] These formulae should not be confused with the usual formulae for probable error — $r = 0.6745 \sqrt{\frac{\Sigma r^2}{n-1}}$ and allied forms used in the method of least squares for the adjustment of observations. Both formulae are developed from the same basic conception of probability and fundamental probability series. However, the least square formulae assume that a fixed value, such as an angle, the distance between two points, etc., is to be ascertained, and that the errors are known to be small, as in the case of a transit for instance, they are known to be less than the smallest graduation on the plate.

The formulae here given are better adapted to statistical work, where larger and more irregular variations are to be expected, and indeed the quantity sought itself often undergoes changes during the investigation. They make use of mathematical conceptions of expectation, and approach more closely to an evaluation of the laws of chance than do the older and more familiar formula.

vational data. If the colon bacilli could be picked out and enumerated with certainty in the sample selected for testing, the result would be subject to error due to chance selection of the sample, this error being the same that must be reckoned with in the determination of morbidity and mortality rates, wherein the distribution of positive and negative cases are governed by laws of chance beyond control of the investigator; but their actual number can be determined by direct count. Here, however, the number of *B. coli* is estimated from the proportion of positive tests, involving an additional source of error, in the determination of this proportion. To determine P accurately requires a large number of tests, in order that all the possible combinations of *B. coli* and water droplets (as determined by the various terms of $(p + q)^s$) may have a fair chance to influence the result in proportion to their probability of existence.

Assume each cubic centimeter to be composed of s minute particles of which a are colon bacilli and B are droplets of water, other bacteria and matter; so that $a + B = s$. Then the probability that any one particle withdrawn in the sample for analysis is a colon bacillus is:

$$p_c = \frac{a_c}{s}$$

and by the same reasoning as before

$$q_c = 1 - p_c$$

Now it can be shown that the most probable or expected number of *B. coli* in a sample of s particles, or

$$e\,(a_c) = sp_c$$

and further, that the *expected error* of this expected value is:

$$\epsilon(a_c) = \sqrt{spq}$$

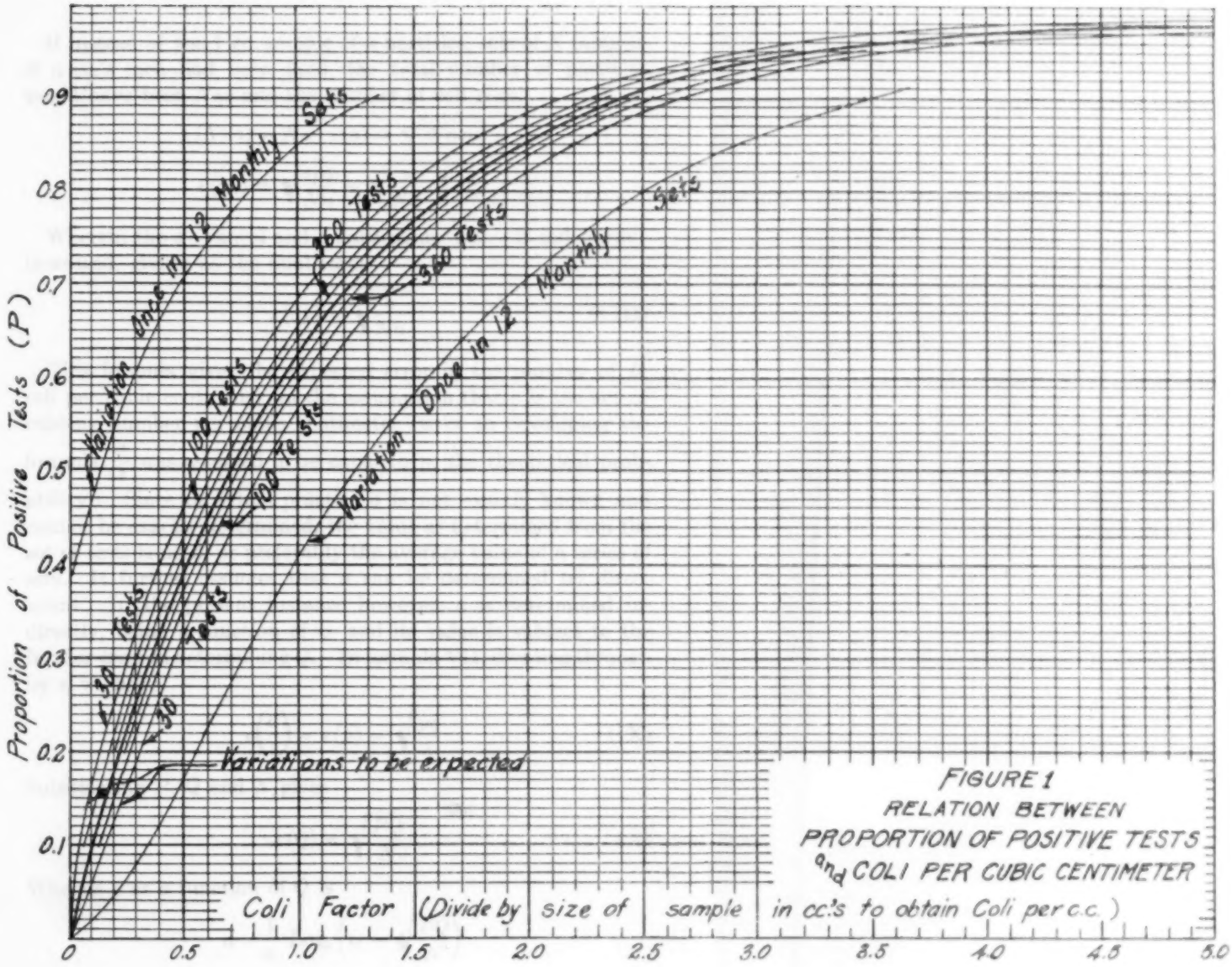
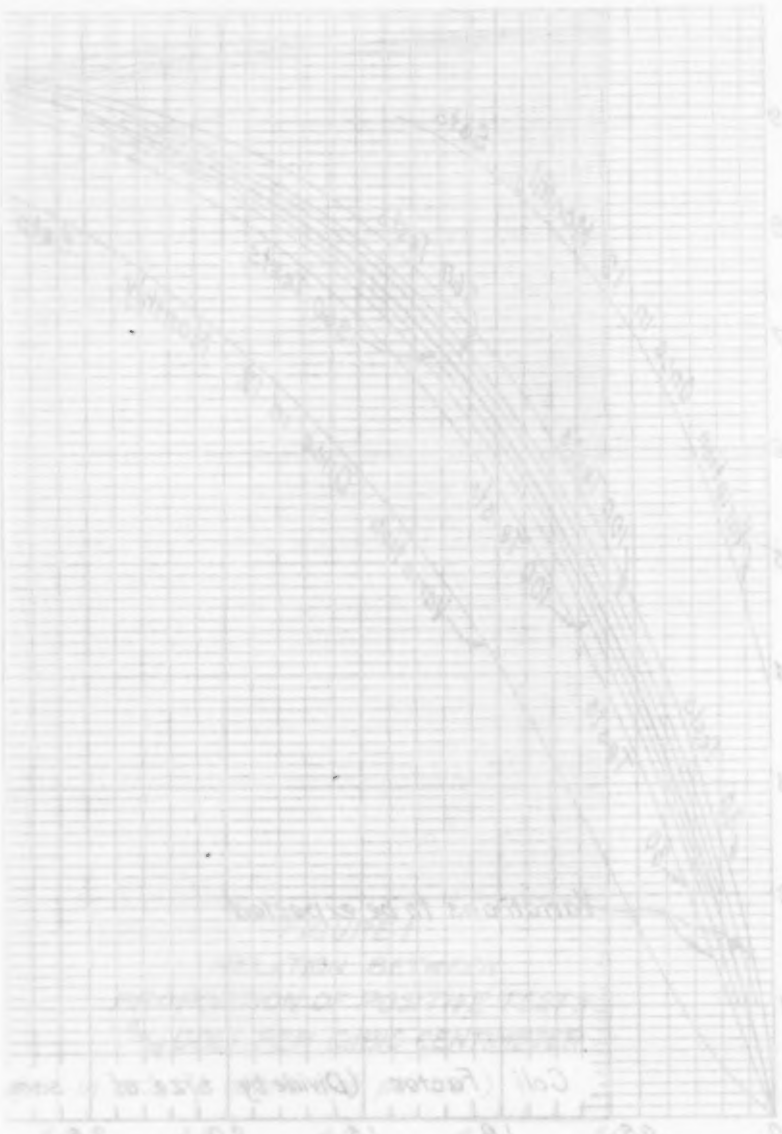


FIGURE 1
RELATION BETWEEN
PROPORTION OF POSITIVE TESTS
and COLI PER CUBIC CENTIMETER



If instead of the 1 cc. sample of s particles, sets of N samples of n cc.'s each had been used, the total number of particles would have been Nns and the number of coli Nna.

$$\epsilon(Nna) = Nn \cdot \epsilon(a) = \sqrt{Nnspq}$$

$$\epsilon(a_N) = \sqrt{\frac{spq}{Nn}} = \sqrt{\frac{s}{Nn} \cdot \frac{a}{s} \cdot q}$$

Wherein the s's cancel and q approaches 1 as s is indefinitely increased, giving as the final form

$$\epsilon(a_N) = \sqrt{\frac{a}{Nn}} \qquad \text{(VIII)}$$

This formula gives the expected error in the number of *B. coli* per cubic centimeter on the assumption that a is the actual existing number per cubic centimeter (since in developing the formula $\frac{a}{s}$ was assumed to be equal to p, the theoretical probability.) Since in actual practice a is not exactly known and cannot be exactly determined, the value as determined from the set of tests is used, or preferably the average value of a series of sets. It further assumes that a can be determined by direct count. In the present instance however, a is determined indirectly, being a function of Q, and its value is subject to the further error in determining Q. In formula VII, dividing through by s, we have:

$$\epsilon\left(\frac{a}{s}\right) = \epsilon(p) = \sqrt{\frac{pq}{s}} \qquad \text{(IX)}$$

Substituting P, Q and N gives

$$\epsilon(Q) = \sqrt{\frac{PQ}{N}} \qquad \text{(X)}$$

Whence a as a function of Q is

$$a = \frac{1}{n} \operatorname{Log}_e\left(Q - \sqrt{\frac{PQ}{N}}\right)$$

Substituting in VIII gives

$$\epsilon(a_N) = \pm \sqrt{\frac{\log_e\left(Q - \sqrt{\frac{PQ}{N}}\right)}{N\,n^2}} \qquad \text{(XI)}$$

which is the formula for the expected error in the number of *B. coli* per cubic centimeter as computed from the value of Q found in a single set of N tests of n cc's each (n being a whole number or fraction). It assumes Q to be correctly known. As this is impossible the next best thing is to take Q as the average of a number of sets of tests.

From formula XI the errors for $N = 30$, 100 and 360 corresponding to series of tests of one month's, one hundred days' and one year's duration, have been computed and plotted in figure 1, assuming one cubic centimeter samples, or $n = 1$. For other values of n the error, as given must be divided by n to obtain the proper value. In this way the range in values corresponding to different proportions of positive tests is visualized.

The frequency with which errors larger than the expected error may occur is given by the formula:

$$T = 1 - \frac{1}{\lambda^2} \qquad \text{(XII)}$$

where λ is the ratio of the error in question to the expected error. Thus the probability of an error not over twice the expected is

$$T_2 = 1 - \left(\frac{1}{2}\right)^2 = 0.75$$

or in other words, there is one chance in four that the error, will be over twice the expected error. In figure 1, curves have been plotted giving the errors which may be expected once in twelve monthly sets and once in four yearly sets.

LIMITATIONS OF THE B. COLI FACTOR THEORY

As is the case with all formulae, whether of theoretical or empirical derivation, the theory herein developed is subject to

certain limitations in its practical application. These limitations have been studied by the aid of the error equations and the following results have been reached.

1. The proportion of positive tests (P) should be based on at least 10 tests; if less are used, the results may be entirely unreasonable due to large errors.

2. The accuracy of results for a given number of tests increases as the value of P increases, so that small values especially below $P = 0.30$ should be avoided if possible.

3. Above about 0.85 the curve, becomes so flat that a small variation in P causes a large variation in the *B. coli* count thus imposing an upper limit.

4. From several points of view a value of $P = 0.65$ gives the closest results for a given number of tests, so that as far as practical, the size of samples should be selected to give approximately this proportion of positive tests.

APPLICATION OF THE B. COLI FACTOR THEORY

In recognition of the frequent mathematical short-comings of bacteriologists, figure 1 has been prepared. By its use it is only necessary to determine the proportion of positive tests in a set, obtain the corresponding factor, from figure 1, and divide this by the size of sample, in order to obtain the *B. coli* per cubic centimeter. The range of variation for the number of samples most commonly used is also shown.

Example:

Data:

Number of tests made, 31.
Number of positive results, 13.
Size of sample, 0.10 cc.

Computations:

$P = 13 \div 31 = 0.42$.
Coli factor corresponding to $0.42 = 0.55$.
$0.55 \div 0.10 = 5.5$ coli per cubic centimeter.

Expected error:

Variation for $P = 0.42$ and $N = 30$ (from figure 1) = from 0.43 to 0.68.

Dividing by 0.10 ($= n$), variation in count = from 4.3 to 6.8.

(6.8 minus 4.3) $\div 2 = \pm 1.25$ = Expected error.

IRREGULAR GROUPING OF POSITIVE TESTS

Every attempt to express the degree of purity of a water supply in terms of the number of colon bacilli is beset with the difficulty that the number of such bacilli varies from day to day. The foregoing theory has assumed that this number is constant while the series of tests was being made, which would place the series in the group known as "normal" mathematically. Actually the *B. coli* content varies in a more or less erratic manner from test to test. This forms a serious obstacle in any attempt to establish a standard of purity, since a series in which all the positive results occur in one group would give the same average as another in which the positive results were evenly scattered, provided the number of positive tests were the same in both cases. By treating a series of tests as if it were a normal series, and then comparing the results with those for a similar number of total and positive tests as given in the foregoing series it is possible to obtain an index of the disturbancy of the series under investigation.

It is usual in a series of observational data to determine the mean, which is the most probable value. By subtracting each observation from the mean the residuals are found which are then squared and summed up. Dividing this summation by the number of observations and extracting the square root gives the *mean error* usually indicated by δ. These operations can be represented by the formula

$$\delta = \sqrt{\frac{(a_1 - M)^2 + (a_2 - M)^2 + \cdots\cdots (a_n - M)^2}{\nu}} \qquad \text{(XII)}$$

δ is the mean error in a as determined by a single set of tests. a_1, a_2, a_3, etc., are the values as determined in the individual

sets of tests. M is the mean or average value of a as determined from v sets of tests. Under ideal conditions (such as were assumed prior to this section of the paper) δ is equal to ϵ of formula XI.

The mean error in a single set (δ) should not be confused with the error of the mean (M) which is

$$\epsilon(M) = \frac{\delta}{\sqrt{v}} \qquad \text{(XIV)}$$

Of course if a large number of tests (say a year's) is considered as a single set then δ cannot be computed and the expected error will be found by the formula for $\epsilon(a)$ and will correspond to $\epsilon(M)$.

The relationship between δ and ϵ for a series of v sets gives us two criteria by which to measure the disturbancy or departure from the ideal conditions of uniform distribution of *B. coli* in the water throughout the tests, assumed in the discussion of a normal series.

The first of these is the Lexian ration L, defined as

$$L = \frac{\delta}{\epsilon} \qquad \text{(XV)}$$

The second of these is the Charlier coefficient of disturbancy

$$100\ p = 100\sqrt{\frac{\delta^2 - \epsilon^2}{M}}\ * \qquad \text{(XVI)}$$

which has the advantage over L of not being affected by variations in the size of the samples.

It will be seen that for normal series $L = 1$ and $100_{\ p} = 0$.

The application of these criteria to the *B. coli* test is best shown by an example:

Data:

Size of samples, 10 cc.
Number of sets, 36.
Number of tests, 360.
Number of positive tests, 32.
Distribution of tests, given in tabulation p. 260.

* The Charlier coefficient is really a measure of the deviation in excess of the expected error for a normal series.

I. Treating these tests as a normal series which assumes that the coli content does not vary during the tests and that the results are only subject to the usual errors of sampling:

a. Proportion of positive tests

$$P = 32 \div 360 \quad = 0.089$$
$$Q = 1.00 - 0.089 = 0.911$$

b. *B. coli* per cubic centimeter:

$P = 0.089$; Factor (from figure 1) $= 0.089$; $\times \frac{1}{10} = 0.0089$ coli per cubic centimeter

c. Expected error in *B. coli* per cubic centimeter:

$$\epsilon(a_N) = \sqrt{\frac{\log\left(0.911 - \sqrt{\frac{0.089 \times 0.911}{360}}\right)}{360 \times 100}} = 0.0018 \text{ per cubic centimeter} = \pm 20 \text{ per cent}$$

II. To take account of the irregular distribution of positive tests first divide the daily tests into sets of ten each; then determine the proportion of positive tests — P — for each set, by dividing the number of positive tests in the set by 10. Find the *B. coli* factor and *B. coli* per cubic centimeter for each set from its value of P. Then find the average coli per cubic centimeter from the 36 sets. By subtracting the value for each set from this average the deviations are found, and from these the mean error. (See p. 260)

Mean error in a single set $(\delta) = \sqrt{\frac{0.038221}{36}} = \pm 0.032 = \pm 248$ per cent

Expected error in a single set $(\epsilon)^* = \sqrt{\frac{.013}{10 \times 10}} = \pm 0.011 = \pm 85$ per cent

$$L = \frac{0.032}{0.011} = 2.9 = \text{Lexian ratio.}$$

$$100\,p = 100\sqrt{\frac{0.032^2 - 0.011^2}{0.013}} = 73 = \text{Charlier coefficient}$$

*Using the formulae $\epsilon(a) = \sqrt{\frac{a}{Nn}}$, where $N = 10$ and $n = 10$.

$$\text{Mean error of the mean } (0.013) = \frac{0.032}{\sqrt{36}} = \pm\ 0.005 = \pm\ 38 \text{ per cent}$$

Thus it is seen that by taking the average for the year (or considering the *B. coli* in the water to be constant) a value of 0.0089 ± 0.0018 was obtained. By arranging the data in a

TABLE 1

The Lexian ratio as a measure of the disturbancy of a year's tests when considered in groups of ten

Number of tests 360
Number of tests in each set 10

LEXIAN RATIO	RATIO OF MAXIMUM B. COLI IN ANY ONE SET TO THE AVERAGE FOR THE YEAR (ASSUMING UNIFORM DISTRIBUTION)					
	P 0.1	*P* 0.2	*P* 0.3	*P* 0.4	*P* 0.5	*P* 0.6
0	1.0	1.0	1.0	1.0	1.0	1.0
0.5	1.2	1.1	1.0	1.0	1.0	1.0
1.0*	2.0	1.5	1.25	1.1	1.0	1.0
1.5	3.5	2.1	1.8	1.5	0.3	1.2
2.0	5.3	3.0	2.4	2.0	1.8	1.6
2.5	8.0	4.0	3.2	2.8	2.4	2.0†
3.0	11.0	5.5	4.0	3.5	3.1†	2.7
3.5	14.5	7.3	5.0	4.5†	4.0	3.5
4.0	18.0	9.0	6.2†	5.4	4.9	4.3
4.5	23.0†	11.5†	7.5	6.3	5.9	5.0

Values of Lexian ratio which will allow a variation from the average for the year as indicated

P	1.5:1	2.0:1	2.5:1	3.0:1
0.1	0.75	1.00	1.20	1.30
0.2	1.00	1.40	1.75	2.00
0.3	1.25	1.75	2.05	2.40
0.4	1.50	2.00	2.30	2.70
0.5	1.75	2.20	2.60	2.90
0.6	1.95	2.50	2.80	3.20

*This line gives the normal error distribution.

†These values correspond to $P = 0.9$ in all the sets containing positive results and therefore represent the above ratio for the most unbalanced grouping possible with sets of ten tests each.

Distribution in daily tests for one year

SET	P	B. COLI FACTOR	B. COLI PER CUBIC CENTIMETER	DEVIATION	(DEVIATION)^2
1	0.0	0.0	0.0	−0.013	0.000169
2	0.1	0.1	0.01	−0.003	0.000009
3	0.0	0.0	0.0	−0.013	0.000169
4	0.1	0.1	0.01	−0.003	0.000009
5	0.2	0.22	0.22	0.009	0.000081
6	0.1	0.1	0.01	−0.003	0.000009
7	0.0	0.0	0.0	−0.013	0.000169
8	0.0	0.0	0.0	−0.003	0.000009
9	0.4	0.5	0.05	0.037	0.001370
10	0.0	0.0	0.0	−0.013	0.000169
11	0.1	0.1	0.01	−0.003	0.000009
12	0.1	0.1	0.01	−0.003	0.000009
13	0.0	0.0	0.0	−0.013	0.000169
14	0.0	0.0	0.0	−0.013	0.000169
15	0.0	0.0	0.0	−0.013	0.000169
16	0.0	0.0	0.0	−0.013	0.000169
17	0.7	1.2	0.12	0.107	0.011450
18	0.8	1.6	0.16	0.147	0.021600
19	0.2	0.22	0.22	0.009	0.000081
20	0.0	0.0	0.0	−0.013	0.000169
21	0.0	0.0	0.0	−0.013	0.000169
22	0.0	0.0	0.0	−0.013	0.000169
23	0.0	0.0	0.0	−0.013	0.000169
24	0.1	0.1	0.01	−0.003	0.000009
25	0.0	0.0	0.0	−0.013	0.000169
26	0.0	0.0	0.0	−0.013	0.000169
27	0.0	0.0	0.0	−0.013	0.000169
28	0.0	0.0	0.0	−0.013	0.000169
29	0.1	0.1	0.1	−0.003	0.000009
30	0.0	0.0	0.0	−0 013	0.000169
31	0.0	0.0	0.0	−0.013	0.000169
32	0.1	0.1	0.01	−0.003	0.000009
33	0.0	0.0	0.0	−0.013	0.000169
34	0.0	0.0	0.0	−0.013	0.000169
35	0.1	0.1	0.1	−0.003	0.000009
36	0.0	0.0	0.0	−0.013	0.000169
Mean.....		0.13	0.013		0.038221

series of sets so as to take account of fluctuations a value of 0.013 ± 0.005 resulted, or 1.7 times as large. The values of L and 100 p show a large disturbancy.

In checking the distribution by this method the number of tests assumed as one set has some effect on the results. In general the number of tests in a set should be the reciprocal of the proportion of positive tests to the total tests for the year, provided however, that the number of tests per set should not be less than ten. The proportion of positive tests in any set should not exceed 0.85, owing to the uncertainty of the *B. coli* factor for larger values. If a large number of consecutive positive tests occur, the series cannot be studied in this manner, since the results are then indeterminate. Such a condition would rather reflect on the analyst's judgment in selecting the size of samples, if he is following the methods given in this paper. It should be noted that it is not necessary to use the same size of sample throughout the year since each set of ten is treated independently. The size should be varied from set to set as required to maintain values as near the optimum as possible.

A careful investigation of these criteria leads the writer to believe that the Lexian ratio is the more useful of the two in studying the *B. coli* test. When L is equal to 1.0 the size of the errors and their distribution is such as may reasonably be expected in sampling a liquor of constant composition. When L is equal to 0.0 the distribution of results is absolutely uniform. Therefore, series in which L is fractional or unity, with perhaps an upper limit of 1.1 or 1.2 may be used with confidence in deriving averages, etc. For the higher values the inequality of distribution and size of errors increases very rapidly (about as L^2) and such series should be discarded for statistical work.

Table 1 gives some results based upon studies similar to the example just considered and will assist in giving a better understanding of the Lexian ratio.

THE "B. COLI CHARACTERISTIC"

The method of working up a year's results so as to find the mean, expected error and standard deviation has just been explained. It will now be shown how these data can be com-

bined so as to give by one figure a rational expression of the *B. coli* content, involving all these factors. Since the proposed form of expression gives the complete *B. coli* condition of the water for the period covered by the tests, it may safely be used in setting standards for purity, etc.

The basic idea is that the information most desired from the results of a series of *B. coli* tests is not an average value, but the maximum *B. coli* which are to be expected in any one test of the series, since it is this maximum value, which must be reckoned with as a factor in considering the possibility of transmission of the water-borne diseases. A water may show very low results for 364 days, but if it contains a large number of *B. coli* on one day of the year, it would be classed as dangerous although the average *B. coli* content would seem quite satisfactory. The actual maximum value obtained in any one test is not necessarily the maximum value to be expected and it is the latter in which we are interested.

By methods similar to those used in developing the expected error, it may be shown that the dispersion in a Lexian series is:

$$\delta_L^2 = \epsilon^2 + \frac{n^2s^2 - ns}{N} \cdot \Sigma \cdot (P_\nu - P_o)^2$$

Where P_ν is the probability in any set, and varies from set to set, and P_o is the average of all the various P_ν's. Substituting for P_ν and P_o the corresponding expression $\frac{a_\nu}{s}$ and $\frac{a_o}{s}$ we obtain

$$\delta_L^2 = \epsilon^2 + \frac{n^2s^2 - sn}{N} \Sigma \left(\frac{a_\nu}{ns} - \frac{a_o}{ns}\right)^2 \text{ or}$$

$$\delta_L^2 = \epsilon^2 + \frac{n^2s^2 - ns}{n^2s^2} \times \frac{\Sigma (a_\nu - a_o)^2}{N}$$

s, being the number of particles in a cubic centimeter, is very large, so that the factor

$$\frac{n^2s^2 - ns}{n^2s^2} \text{ or } 1 - \frac{1}{ns}$$

may be considered equal to unity. The last term is then the expression for the *standard deviation*, or

$$\delta_L = e^2 + \delta^2$$

Where δ_L may be considered as the total expected error in any *one* set of those, comprising the series.

Assuming a year's tests to be composed of 36 sets of ten, the error to be expected once in 36 times is found by the criterion of Tchebycheff:

$$1 - \left(\frac{1}{\lambda}\right)^2 = \frac{35}{36}, \text{ whence } \lambda = 6$$

so that one set of ten may be expected to have a deviation of $6\ \delta_L$.

Since the expected error is proportional to the square root of the number of tests, that for a single test in a set of ten would be the error in a set multiplied by $\sqrt{10}$ or 3.16. This gives as the final result

$$\delta_L = 3.16 \times 6 \times \sqrt{e^2 + \delta^2} = 18.96 \sqrt{e^2 + \delta^2}$$

and adding this to the average value for the coli, gives

$$C\,\frac{360}{10} = a_{10} + 18.96 \sqrt{e\,(a_{10})^2 + \delta_{a10}{}^2}$$

$C\,\frac{360}{10}$ is the maximum number of *B. coli* per cubic centimeter to be expected once in 360 tests, these tests being considered in sets of ten. It is also a very concise and rational manner of stating the *B. coli* data relative to a water, since it combines in a logical and correctly weighted form, the average value, expected error and the standard deviation due to fluctuations in the water from test to test. For these reasons it may be called the *B. coli* characteristic. It is easily applied, since a_{10}, $e\ (a_{10})$ and $\delta_{a_{10}}$ are readily obtained by the methods given in previous

sections of this paper, and their combination involves only a simple computation. Applying this to the previous example, where:

$$a_{10} = 0.013$$
$$e\,(a_{10}) = 0.011$$
$$\delta\,(a_{10}) = 0.032$$

gives

$$C\frac{360}{10} = 0.013 + 18.96\sqrt{0.011^2 + 0.032^2} = 0.668$$

In its general form, this formula is

$$C_{\frac{N_p}{N}} = a_N + \sqrt{N_p\,(e\,(a_N)^2 + \delta a_N{}^2)}$$

the symbols having the usual meaning.

Commenting on the foregoing method of interpreting a year's tests, the first question naturally is whether ten tests are sufficient to serve in computing the percentage of positive tests. It is a rather small number for such a purpose, but even so, the ten day period embraced in each set of ten tests, is already longer than desirable to allow the daily variations to assert themselves in the average. Therefore, if this method is to be accommodated to the routine practice of one test daily, the ten day period is probably as good as can be chosen. We must then expect an error of from 10 per cent for 0.9 positive to 100 per cent for 0.1 positive and averaging probably 25 per cent; this error being added into the *B. coli* characteristic and thus being taken into account. Selection of a size of sample to give positive results in the neighborhood of 0.65 will assist greatly in securing reliable results, and if more accurate data are desired, a series of ten fermentation tubes can be run every day, since with proper arrangements this would require little additional time.

In deriving a *B. coli* value from a series of tests, the assumption is that the *B. coli* content remains constant during the series, which is not actually the case. The factors causing change are

numerous and include: river stages, wind velocity and direction, temperature conditions, season of year, time of day, etc. Probably the various combinations of these conditions are very numerous. On the other hand, it must be recognized that there are equalizing tendencies due to the time element and to dilution. Such damping effects play a much more important part in the case of tests on water supplies drawn from rivers and lakes, than in the case of tests on crude sewage. If a sufficiently large number of tests are taken the variation from set to set as given by the standard deviation should cover as well the range in values to be expected from test to test. The 36 sets obtained in a year's tests are probably insufficient but must be used as the best data available.

It should be realized that even with 360 tests this method gives results that are far from exact, but they are along rational lines and can be increased in accuracy by increasing the number of tests.

SIGNIFICANCE OF REPEATED POSITIVE RESULTS

Considerable significance attaches to repeated positive tests when using a size of sample which gives a low percentage of positive tests. If 10 per cent of the tests are positive or $P = 0.1$, then the probability of obtaining two consecutive positive tests would be $\frac{1}{10} \times \frac{1}{10}$ or 0.01: and for three consecutive positive tests, $\frac{1}{10} \times \frac{1}{10} \times \frac{1}{10}$ or 0.001. In general if a percentage of positive tests, P, has been established by a series of tests on samples of a certain size, the probability of obtaining two consecutive positive tests is P^2 and of three such tests P^3, etc. This affords a ready means for detecting sudden increases in the number of *B. coli* or, in investigating old records, assists in identifying periods of more than the average pollution.

THE USE OF THE NITRATE-REDUCTION TEST IN CHARACTERIZING BACTERIA

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INTRODUCTION

For some time bacteriologists have used the ability to reduce nitrates as a criterion for distinguishing certain kinds of bacteria. The test has been regarded as of so much diagnostic value that it has been included in nearly all schemes of bacterial characterization, among the most important of which is the descriptive chart adopted by the Society of American Bacteriologists. An investigation of this test was begun as a result of some student work done under the direction of one of us (B) upon certain cultures of the colon group. Such very irregular results were obtained in regard to the power of reducing nitrates that the method of making the test was decided to be unsatisfactory. Further work was therefore undertaken, at first by students and volunteer assistants, and then by one of us (C) as a contribution to the work of the Committee on the Descriptive Chart appointed by the Society of American Bacteriologists. For the earlier part of the work much credit is due to Emma Edson Breed, H. M. Weeter, and H. V. Grant.

TECHNIC

1. Formula of medium. An attempt was made at first to follow the "standard" technic adopted by the American Public Health Association (1905, 1912). It was soon found, however, that a standard technic was sadly lacking. In the 1905 Report of the Committee on Standard Methods of Water Analysis the following directions are given:

Dissolve 1 gram peptone in 1 liter of tap water, and add 2 grams of nitrite-free potassium nitrate. It is convenient to prepare a stock solution of potassium nitrate by dissolving 4 grams of solid nitrate in 100 cc. of distilled water and use 5 cc. of this solution in the above formula.

A little arithmetic will show that to follow the direction given in the first sentence would give a 0.2 per cent solution, while that in the second sentence would give a 0.02 per cent solution of the nitrate. In the 1912 report an attempt was evidently made to correct this disagreement, because the two grams in the first sentence was changed to 0.2 gram; but by some slip the 4 grams in the second sentence was also changed to 0.4 gram, thus giving a 0.002 per cent solution. With this inconsistency there has naturally been a great variation in the "standard" nitrate broths used by different bacteriologists. This is shown by a survey of the literature. Thus Gorham (1901) recommends 1 gram of peptone and 0.2 gram of potassium nitrate (i.e., 0.02 per cent); while Chester (1901) recommends a formula different from any of the above—10 grams of peptone and 0.02 gram (i.e., 0.002 per cent) of *sodium* nitrate. Apparently in preparing a formula for nitrate broth the figures 1 for the peptone and 2 for the nitrate have been considered more important than the position of the decimal point in either case. This peculiar irregularity has already been mentioned by one of us (Breed, 1915).

In the early part of this work two different nitrate broths were used independently by two of the workers, each thinking he was following the standard technic; both contained 0.1 per cent peptone but one contained 0.2 per cent KNO_3 and the other 0.02 per cent KNO_3. When the discrepancy was noticed, the two media were compared, as were other different nitrate media, all of them within the limits of variation of the formulae given above. Later, other arbitrary variations were made for special purposes, as will be explained later.

2. The nitrite test. Two different variations of the official nitrite test were used in the first part of this work, differing only in the quantity of the reagents used. Each variation was sup-

posed to be "standard" by the particular investigator employing it, owing to two different interpretations of the standard methods. On page 120 of the report for 1912 the following tchnic is given: "Remove 3 cc. of the culture to a clean test tube and add 2 cc. of each of the naphthylamine solution and the sulphanilic acid solutions described under the determination of nitrites (see p. 22)." Three of the workers followed these directions exactly. Another, working independently, looked up page 22 and found that at this place the directions were to add 2 cc. to 100 cc. of the water to be tested, and concluded that the proportions of reagents given on page 120 must have been a mistake; so he used the reagents in the proportion of about 2 parts to 100 parts of the medium, i.e., a few drops to the culture tube. Later these two methods of using the reagents were compared, and the latter method was found to be the better. Using larger quantities of the reagent was found to dilute the nitrite present to such an extent as to obscure the reaction in cases where only small amounts of nitrite had been formed, especially when the reagents were old and somewhat discolored. As a result, in the latter part of the work, only a few drops of each reagent were added to the culture.

A still different test was used in some of the early part of the work: the potassium-iodide-starch test, as described by Erwin Smith (1905). This test compared very favorably with the official test, but proved a little less delicate and the reagents were found to deteriorate more rapidly; so its use was discontinued. This test is preferred by Harding (1910) just because it is less delicate; but as the present work progressed, the need of a delicate nitrite test was emphasized more and more clearly.

WORK WITH THE COLON GROUP

In 1912, one of the students taking part in the work (E. E. B.) observed considerable irregularity in the nitrate-reduction test in the case of fifty different cultures of the colon group isolated from polluted water. These cultures included all four of the commonly recognized types, as distinguished by their fermen-

tative reactions with sucrose and dulcite. They were tested in triplicate in 0.02 per cent nitrate broth (with 0.1 per cent peptone), some giving negative results in all three tubes, others giving inconsistent results, while the majority of the cultures gave consistent positive results as was expected. In 1913 the cultures giving no positive reactions the first time were tested again by the same student, using the same medium, and all but three of the cultures were found to produce nitrite in at least one out of three tubes. These three cultures and one other (which had shown nitrite in only one tube each time) were tested a third time with consistently positive results. These findings are listed in table 1 under the heading "Series of 1912–13." In all, about 64 per cent of the tests were positive, counting each tube in a set of triplicates as a single test. The fact that every culture gave a positive reaction in the end suggested that all might be nitrate-reducers, but that the methods of testing were such that they did not develop this ability in more than about two-thirds of the cases.

Later in 1913 (see table 1, Series of 1913) the same cultures were tested in triplicate again by another student (H. M. W.) using the same medium and the same methods generally, recording the results similarly. By this student 37 cultures were tested twice, 15 of them three times. Until the formula of the medium was varied, only about 37 per cent of the tests were positive. In regard to the individual cultures, there was practically no agreement with the results of the first student: only three cultures (nos. 11, 14 and 35) gave consistently positive reactions on both occasions.

In the course of this work it was noticed that no strain grew well in the medium used; so some preliminary work was done in varying the composition of the medium (last column under "Series of 1913," table 1). Twelve cultures that had given negative reactions in the majority of cases were tested again in a medium containing 0.2 per cent (instead of 0.1 per cent) peptone. The growth in this medium was noticeably better, and a distinct nitrite reaction was obtained in all 36 tubes.

TABLE 1

Nitrate reduction by organisms of the colon group in 0.02 per cent nitrate broth. Tests made in triplicate; + and − indicate presence and absence respectively of a distinct nitrite reaction in all three tubes; ± indicates a distinct nitrite reaction in two out of the three tubes, ∓ in only one of the three tubes. T indicates mere trace of nitrite.

CULTURE NUMBER	SERIES OF 1912-13			SERIES OF 1913				SERIES OF 1914		
	Peptone 0.1%			Peptone 0.1%			Peptone 0.2%*	Peptone 0.1%	Peptone 0.2%	Peptone 0.5%
	First test	Second test	Third test	First test	Second test	Third test				
1	−	+		−	−	+				
2	−	∓		−	∓			∓	+	+
3	+			−	+			∓	+	+
4	±	+		−	±			−	+	+
5	−	+		−	+†			±	+	+
6	±	±		−	T	+		−	+	+
7	+			−	−	+	+	+	+	+
8	−	+		±				−	+	+
9	+			−	T‡			−	+	+
10	−			−	∓			±	+	+
11	+			+	+			+	+	+
12	±			−	−	+		∓	+	+
13	+			−	±			+	+	+
14	+			T	+					
15	+			±				+	+	+
16	T?	+		−	T	−	+	+	+	+
17	−	+		−	+					
18	±			±				−	+	+
19	+			−	±†			−	+	+
20	−	+		−	∓	−	+	−	+	+
21	±	+		+				−	+	+
22	−	+		−	∓			+	+	+
23	+			−	T	+		±	+	+
24	+			−	±			−	+	+
25	−	+		−	+†			+	+	+
26	−	+		−	−	+	+			
27	∓	+		−	+			−	+	+
28	+			±				∓	+	+
29	+			−	−†	+	+	∓	+	+
30	+			−	∓			±	±	+
31	−	+		−	−	±	+	−†	+	+
32	+			∓	+			−	+	+
33	+			−				−	±†	+
34	−	+		−	+	+	+	±	+	+
35	+			+				+	+	+

TABLE 1—*Continued*

CULTURE NUMBER	SERIES OF 1912-13			SERIES OF 1913				SERIES OF 1914		
	Peptone 0.1%			Peptone 0.1%			Peptone 0.2%*	Peptone 0.1%	Peptone 0.2%	Peptone 0.5%
	First test	Second test	Third test	First test	Second test	Third test				
36	+			−	+			+	+	+
37	+			−	−	+	+	∓	+	+
38	−	+		−	−	−	+	±	+	+
39	+			−	−	+	+	−	+	+
40	±			−	−			−	+	+
41	−	−	+	+	±†			+	+	+
42				−	T	−	+	−	+	+
43	−	−	+	−	+			−	+	+
44	+			−	+	+	+	+	+	+
45	−	−	+	+	−	?	+	±	+	+
46	∓	∓	+	+				−	+	+
47	+			∓	T			−	+	+
48	+			+	±			±	+	+

* This medium contained only 0.01 per cent nitrate.
† One of the tubes showed nitrite present in mere traces only.
‡ One of the tubes showed a distinct positive reaction.

The idea suggested by the last mentioned tests was followed up by a third student (H. V. G.) the next year. Parallel tests were made in nitrate broth containing 0.1, 0.2, and 0.5 per cent peptone respectively (Series of 1914, table 1). With 0.1 per cent peptone only 42 per cent of positive results were obtained, and the agreement of the individual cultures with their previous behavior was no greater than it had proved to be the preceding year. With 0.2 per cent peptone 98.5 per cent of the tests (129 out of 131 tubes) gave positive results, every culture showing a positive reaction in at least two of the tubes. With 0.5 per cent peptone all of the cultures gave positive reactions in all three of the triplicate tubes. Further tests not listed in the table were made with a medium containing 0.1 per cent peptone and 0.2 per cent (instead of 0.02) per cent nitrate. This use of ten times the original amount of nitrate was found to have no influence on the results, the irregularity proving as great as with 0.1 per cent peptone and 0.02 per cent nitrate.

The conclusion reached by this work was that inconsistent results of the nitrate-reduction test may be expected with organisms of the colon group unless enough peptone is present to furnish these bacteria with conditions favorable to their vigorous growth. With 0.1 per cent peptone the growth was generally poor; with 0.2 per cent fairly good; and with 0.5 per cent very good. The amount of nitrate present apparently had little influence on the results. The important matter was a vigorous growth of the organisms; and under conditions allowing vigorous growth all the cultures of the colon group tested proved to reduce nitrate to nitrite.

WORK WITH BACILLUS CEREUS FRANKLAND

As an organism to contrast with those of the colon group, *B. cereus* Frankland[1] was chosen. *B. cereus* grows well in the presence of considerable organic matter, but it does not seem to require the large amounts of nitrogenous material that the colon organisms do. The cultures of *B. cereus* used were isolated by one of us (C) from soil.

Ten out of 130 cultures, apparently all *B. cereus*, failed to produce nitrite when tested, promptly after isolation from soil, in broth containing 0.1 per cent peptone and 0.2 per cent KNO_3. One of these ten cultures (which we will denote culture x) was tested again two years later (table 2, test 2) together with several typical *B. cereus* cultures (which we will call cultures A to G respectively) that had produced nitrite the first time. This work was done by a different investigator (H. V. G.) from the one who made the original tests. In order to see whether the explanation for the disagreement previously found might be due to the same cause as the disagreement in the case of the colon organisms, the four following nitrate broths were used:

0.1 per cent peptone, 0.2 per cent nitrate
0.1 per cent peptone, 0.02 per cent nitrate
0.2 per cent peptone, 0.02 per cent nitrate
0.5 per cent peptone, 0.02 per cent nitrate

[1] This organism was identified by means of the characteristics previously described (Conn 1917).

TABLE 2

Nitrite production by B. cereus in various nitrate media. Tests made in duplicate. Each + or − sign indicates presence or absence of nitrate in all three tubes of one set. The sign ± indicates disagreement among three parallel tubes

	ORIGINAL TEST 1911-13	SECOND TEST, 1914				THIRD TEST, 1914			FOURTH TEST, 1914	FIFTH TEST, 1915						SIXTH TEST, 1918		SEVENTH TEST, 1918	
Medium containing KNO_3	0.2%	0.2%	0.02%	0.02%	0.02%	0.2%	0.2%	0.2%*	0.2%	0.2%	0.2%	0.2%	0.02%	0.02%	0.02%	0.2%	0.1%†	0.1%†	0.1%‡
Medium containing Peptone	0.1%	0.1%	0.1%	0.2%	0.5%	0.1%	0.5%	0.5%	0.1%	0.1%	0.2%	0.5%	0.1%	0.2%	0.5%	0.1%	1.0%	1.0%	0.0%
Culture A	+	+	+	+	+	+												+	+
Culture B	+	+	+	+	+	+													
Culture C	+	+	+	+	+														
Culture D	+	+	+	+	+														
Culture E	+	+	+	+	+														
Culture F	+	+	+	+	+														
Culture G	+	+	+	+	+														
Culture H	+																	+	+
Culture X	+					−	−	−	−	−	−	−	−	−	−			−	−
Culture Y	+								−										
Culture a	−					−	−	−		−	−	−	−	−	−	−	−		
Culture b	−					−	−	−	−	−	−	−	−	−	−			−	−
Culture c	−					−	−	−	−	−	±	±	−	−	−				
Culture d	−					−	−	−		−	−	−	−	−	−				
Culture x	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+				

* This medium contained 1 per cent glucose.

† Nitrate-peptone agar. Test made on agar slants.

‡ Five different nitrate agars used, containing no added organic matter except sugar. Tests made on agar slants. Each medium inoculated in triplicate and the three tubes tested on different days. Each + or − sign indicates presence or absence of nitrite in all fifteen tubes.

Each culture was inoculated in triplicate into each medium; and clear-cut positive nitrite reactions were obtained in all cases, even with culture x.

A few months later (test 3, table 2) cultures A and B, together with seven other typical nitrite-positive *B. cereus* cultures[2] were tested again in the first of the nitrate broths listed above, all giving such clear-cut positive reactions that no further investigation of these cultures were made. At the same time, six other cultures were retested in this same medium, and also in media of the following composition:

0.5 per cent peptone, 0.2 per cent nitrate;
0.5 per cent peptone, 1 per cent glucose; 0.2 per cent nitrate.

One of these six cultures (culture X, table 2) had given a positive reaction the first time, while the others (cultures a, b, c, d and x) had given negative reactions. The results showed no nitrite from cultures a, b, c, d, and none from culture X, while nitrite was found with culture x, as had been the case in test 2. In other words all the cultures except X and x showed agreement with the original test; but X had changed from nitrite-positive to nitrite-negative, x from nitrite-negative to nitrite-positive.

Test 4 was made a few weeks later. This test included cultures b, c, x and X, together with a fifth, which from its similarity to culture X will be denoted as culture Y. Cultures b, c, x and X gave the same results as in test 3. Culture Y, like culture X, had given a positive nitrite reaction at the time of isolation two years earlier, but now showed no production of nitrite.

No further tests of culture Y were made, but cultures X, a, b, c, d and x were tested again about a month later (test 5). This test was made in six different nitrate broths, the four used in test 2 together with two additional broths as follows:

0.2 per cent peptone, 0.2 per cent nitrate
0.5 per cent peptone, 0.2 per cent nitrate

---

[2] Not listed in Table 2, because no further work was done with these seven cultures.

All the cultures except culture c gave the same results as in the two preceding tests, no difference being observed between their behavior in the different media. Culture c gave distinctly negative results with the four media used in test 2; but with the two new media (that is, the media with 0.2 per cent nitrate combined with more than 0.1 per cent peptone) a discrepancy was observed on the tenth day, one of the two duplicate tubes of each medium tested giving a positive nitrite test.

Four years later culture a was tested again, still with negative results. In the meanwhile the advantage of agar for making the test in doubtful cases (see p. 277) had been learned, and so this culture at this time was tested also on beef-extract-peptone agar to which 0.1 per cent KNO_3 had been added. The results were still negative. Good growth, however, was obtained in all cases.

Test 7 was made a few months later. Three organisms were tested this time: H, a typical nitrite-positive *B. cereus* culture that had not been tested before since immediately after isolation five years before; culture X and culture b. These cultures were tested at this time in six different agar media, all but one of them containing no added organic matter except some sugar. Culture H gave positive results in all the media used; cultures X and b gave negative results throughout although one tube out of nine in the case of culture b gave a positive nitrite reaction on the seventh day (due probably to an impurity). One of the media used contained no possible source of ammonia except the nitrate, so a Nessler test was made to see if the nitrate had been converted into ammonia without accumulation of nitrite. This test also was negative.

As a result of this work it was concluded that with *B. cereus* the explanation of the irregularities is not as simple as in the case of the colon organisms. *B. cereus* grows well in almost any medium and ordinarily reduces nitrate to nitrite. Certain cultures, however, seem to lack this reducing power, either temporarily or permanently, although they grow well in the media used. In general, constantly negative or constantly positive results have been found with any particular culture. This sug-

gests that there may be two different species so closely related that they can be distinguished only by means of the nitrate test. Three cultures, however (X, Y, and x) and possibly a fourth (c) gave inconsistent results. Although this disagreement may possibly have been due to a contamination that ran out the original organism, the agreement in all other respects with the original descriptions makes this explanation doubtful. No other explanation of the irreglarity has been found, however, unless it be assumed that cultures of *B. cereus* may lose or gain the power of reducing nitrates when cultivated in the laboratory.

WORK WITH FLUORESCENT PSEUDOMONADS

During the course of a study of soil bacteria, numerous cultures of fluorescent pseudomonads were obtained that appeared to be closely related although differing in certain particulars. One of the most noticeable points of difference was that some produced nitrite in nitrate broth containing 0.1 per cent peptone while others produced no nitrite under these conditions. With these organisms the growth was always fairly good; nevertheless it was felt that the difference might be due to causes similar to those affecting nitrate-reduction by the colon organisms. Upon testing the cultures in broth containing larger amounts of peptone (as much as 1 per cent) the same distinction between nitrate-producers and non-nitrite-producers held true; but in this broth a further difference appeared, some of the organisms producing gas (presumably free N), others producing no gas. In other words three groups of fluorescent pseudomonads were found upon inoculation into 1 per cent peptone solution containing nitrate: (1) producing nitrite and gas; (2) producing nitrite but no gas; (3) producing neither nitrite nor gas (in appreciable quantities).

As the fluorescent organisms grow rather better on the surface of agar than in a liquid medium, further work was carried on in a nitrate agar containing beef-extract 0.3 per cent, peptone 1 per cent, KNO_3 0.1 per cent. The nitrite test was made by pouring the reagents upon the surface of the agar slant after incubation;

gas was detected by means of bubbles and cracks in the medium. One day of incubation was enough to bring out the nitrite reaction with vigorous nitrate-reducers; but cultures were generally kept until the seventh day. This agar slant test was found to give the same results obtained with the 1 per cent peptone broth cultures; the same three groups were found as before.

TABLE 3

Composition of nitrate media used in tests listed in tables 4–7. Figures indicate grams per liter.

INGREDIENTS	MEDIA SYMBOLS*					
	P	DM	D	DL	S	DA
Agar†	15.0	15.0	15.0	15.0	15.0	15.0
Peptone	10.0					
Glucose		10.0	10.0	10.0		10.0
Lactose				5.0		
Sucrose					10.0	
KNO_3	1.0	1.0	1.0	1.0	1.0	1.0
$CaCl_2$		0.5	0.5	0.5	0.5	0.5
$MgSO_4$		5.0				
K_2HPO_4		5.0	0.5	0.5	0.5	0.5
NH_4Cl						2.0
Beef extract	3.0					

* In these media symbols, the letters represent the significant ingredients, as follows: P, peptone; D, glucose; M, magnesium sulphate; L, lactose; S, sucrose; A, ammonium chloride.

† "Bacto-agar" (a purified agar sold by the Digestive Ferments Co.) was used in all except Medium P.

For further testing six cultures were selected, two of each group. These six cultures will be denoted in this paper as follows:

AA, BB } Producing nitrite and gas in nitrate-peptone media.

A, B } Producing nitrite without gas in nitrate-peptone media.

a, b } Producing neither nitrite nor gas in nitrate-peptone media.

These six cultures were retested not only on the nitrate-peptone agar previously used (test 1, table 4) but also on agar containing no nitrogen (disregarding impurities) except 0.1 per cent KNO_3 and with no added organic matter except sugar (tests 2–5, table 4). The object was to learn whether the nitrate was converted to ammonia. The reports of the Committee on Water Analysis (A. P. H. A. 1905, p. 150; 1912, p. 120) have called attention to the need of making the ammonia test, but have added that ammonia may also come from the peptone. Kligler (1913) emphasized the importance of this source of error. As the fluorescent organisms are all ammonifiers, it is impossible to tell whether the ammonia present in a nitrate-peptone medium comes from the nitrate or the peptone. Hence it was decided to grow them under conditions where there could be no source of ammonia except the nitrate, so that its presence would show reduction of the nitrate even though there was no accumulation of nitrite. The first media tried contained glucose (media DM, and DL, table 3) and were found to be unsatisfactory for the ammonia test because no glucose could be obtained free from ammonia. Lactose was found to have the same disadvantage, so finally sucrose (medium S, table 3) was employed as a source of energy.

Even before a satisfactory ammonia-free medium was obtained, some very interesting results were procured (see table 4). On these media all six cultures, even including strains a and b, were found to give the nitrite test, sometimes in mere traces, but often in appreciable quantities, as early as twenty-four hours after inoculation. The test on the ammonia-free medium S (test 4, table 4) gave the following results: Cultures AA and BB showed the presence of ammonia, as well as the nitrite and gas demonstrated on the peptone media; cultures A and B showed a very strong nitrite reaction, but a weak ammonia reaction after the fourth day; cultures a and b showed a moderate nitrite reaction and an ammonia reaction slightly stronger than with cultures A and B. The ammonia reactions were in no case strong enough to prove that the organisms were converting the nitrate rapidly into ammonia.

TABLE 4

Nitrate reduction by Ps. fluorescens in 0.1 per cent nitrate agar. First test. Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, − absent, ? doubtful.*

	MEDIUM P.		MEDIUM DM.		MEDIUM DL.			MEDIUM S						MEDIUM DA.		
	Nitrite present in		Nitrite present in		Nitrite present in			First day		Second day		Fourth day		Nitrite present in		
	1 day	4 days	4 days	7 days	1 day	2 days	3 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	1 day	2 days	3 days
Culture A	+++	T	−		+++	+++	+++	++	?	+++	−	+++	T	++	++	++
Culture B	+++	+++	−	+	+	+++	+++	++	?	++	−	+++	T	++	++	++
Culture a	−	−	T		+	+	+	+	?	+	+	+	+	−	−	−
Culture b	−	−	T		+	T	T	+	?	+	+	+	+	−	−	−
Culture AA	+	T†	+†		++	++	+++†	+++	?	+++	+	+†	++†			++
Culture BB	++†	T†	−†		+	++†	+†	+	++	+	−	+	T		†	+†

* In this test the five media were inoculated separately, on different days.

† Gas production shown by presence of cracks in the agar.

A further test (test 5, table 4) was then made on a synthetic agar (medium DA, table 3) containing nitrate, glucose, and ammonium chloride. Cultures A, B, a and b gave the same reactions (i.e., either nitrite-positive or nitrite-negative) as on peptone media; of the gas-producers, BB behaved as on the other media, but AA failed to produce gas. In other words, it was shown that cultures a and b were prevented from producing nitrite on the synthetic medium by the addition of ammonium chloride. This test was considered to show that some strains of fluorescent organisms, although capable of reducing nitrate, do not attack it if there is a more easily available source of nitrogen present.[3] In this connection it is interesting to note that the nitrite reaction of these two organisms was stronger on the sucrose medium than on the media containing those sugars which had ammoniacal impurities.

The sixth test (table 5) was run a few months later. This test was a repetition of tests nos. 2 to 5, using the same four media together with one other glucose agar only slightly different (medium D, table 3). The same six cultures were tested, together with four others (laboratory cultures isolated from soil nearly six years previously): two gas-formers CC and DD, and two that were nitrite-negative in peptone media, c and d. Of these four cultures, DD was especially interesting. It had been kept under cultivation in the laboratory for the longest time of all, and when first tested on medium P (nitrate-peptone agar) six years after isolation, was found to be a weak gas-producer. During the next few months its power of gas-production apparently diminished until when the work recorded in table 5 was done it failed to produce gas on any medium. The possibility of a contamination being present and causing this discrepancy is not entirely excluded.

The four cultures AA, BB, A, B, a, and b, gave results agreeing fairly well with the earlier tests, except in regard to the gas-

[3] Another possible explanation is that growth is so rapid in the presence of ammonium chloride that these organisms are able to use up the nitrite as fast as produced. This theory is not a satisfactory explanation, however, because the growth of Cultures a and b was better in the absence than in the presence of ammonium chloride in this medium.

TABLE 5

Nitrate reduction by Ps. fluorescens in 0.1 per cent nitrate agar. Second test. Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, − absent, ? doubtful*

CULTURE	MEDIUM DM.				MEDIUM D.				MEDIUM DL.				MEDIUM S								MEDIUM DA.			
	Nitrite present in				Nitrite present in				Nitrite present in				2 days		4 days		5 days		7 days		Nitrite present in			
	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	2 days	4 days	5 days	7 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	2 days	4 days	5 days	7 days
A.....	+++	++	+++		+++	+++	+++		+++	+++	++	++	+++	T	++	?	++	T			+++	+++	+	
B.....	+++	+++	+++		+++	+++	+++		+++	+	++		+++	T	++	?	++	T			++	+	+	
a.....	+	T		+	+	+	+	T	+	T		+	++	+	++	?			++	?	−	−		−
b.....	+	T		+	+	+	+	T	+	T		T	++	+	++	?			++	?	−	−		−
c.....	−		−	−	−		−	−	−		−	−	−	T			−	T	−	−	−		−	−
d.....	−		−	−	−		−	−	−		−	−	+	−			+	+	T	T	−		−	−
AA.....	++	−†	−†		++	++	++		++	++	++†		+++	+	T†	+	−†	+			++	++	++	
BB.....	+++†	+†		T†	+++	+†	T†		+++	+++†	−†		+	T	+	T	+	T			+++†	+++†	+++†	
CC.....	+++	+†	T†		+++	+++†	+†.		+++	+++†	+++†		+	T	+	T	+	+			++	+++†	+++†	
DD.....	+	T		+	+	−		−	+	T		−	+	−	+	−			+	−	−	−	−	

* In this test the five media were inoculated simultaneously.

† Gas production shown by presence of cracks in the agar.

production by AA and BB on certain media, a point which seems open to considerable variation. The two old cultures, c and d were found to be even less vigorous nitrate-reducers than a and b. Culture c produced no nitrite on any medium and only a trace of ammonia on medium S; culture d showed nitrite and ammonia on medium S, but no nitrite on any other medium. Culture c grew very poorly on medium S, a fact which undoubtedly explains its failure to reduce the nitrate on this synthetic medium.

It was therefore concluded that vigorous growth of the fluorescent organisms is as necessary as it is with the colon organisms, in order to get a satisfactory test for nitrate-reduction; but that a more common cause of error is that cultures do not always reduce the nitrate if a more readily available source of nitrogen is present. In the present work the difference between those cultures that could reduce nitrate under all conditions and those that could do so only in the absence of other sources of nitrogen remained constant; but, as the two sets of cultures resembled each other in all other respects and as various gradations were found between those that showed no nitrite in the presence of mere traces of ammonia and those that produced it even in the presence of peptone, it does not seem to be a question of two distinct species.

WORK WITH AN ORANGE CHROMOGENIC PSEUDOMONAS

Another organism chosen for study because of its irregularity in respect to the nitrite test was an orange chromogenic pseudomonas, a form very common in soil and water. It has recently been studied in this laboratory and a description of it is now in course of publication (Conn and Bright, 1919). It is concluded to be identical with *Bacillus caudatus* Wright (1895), although it has a polar flagellum. This organism grows so poorly in liquid media of all sorts that nitrate broth was realized from the beginning to be an unfair medium to use in the nitrate-reduction test, and the early irregular results were considered to be due to the poor growth in this broth. For that reason beef-

extract-peptone agar containing 0.1 per cent nitrate (medium P, table 3) was used in subsequent work.

A special study was first made of eight different strains of this organism, four of which strains had been separated into two or three substrains immediately after isolation from the soil, all of these substrains having been kept distinct during the laboratory cultivation. Including these separate substrains, 15 different cultures were studied. It was found (see test no. 1, table 6) that they could be divided into two groups, those producing abundant nitrite on nitrate-peptone agar and those showing no nitrite on this medium. The cultures may be listed as follows:

Group 1.—Producing nitrite

A. A typical chromogenic strain.
B. A typical chromogenic strain.
C_1, C_2 } Two substrains of a typical chromogenic strain.
D_1, D_2, D_3 } Three substrains of a typical chromogenic strain.
X_1, X_2, X_3 } Three substrains of a non-chromogenic strain.

Group 2.—Not producing nitrite

a_1, a_2, a_3 } Three substrains of a chromogenic strain.
b A chromogenic strain.
c A chromogenic strain

On nitrate-peptone agar the agreement between the different substrains of any one original strain was complete. Some of the strains grew more vigorously than others. In general there was no correlation between vigor of growth and production of nitrite, although it was striking that after a few months cultivation in the laboratory all the nitrite-negative cultures died.

TABLE 6

Nitrate reduction by the orange chromogenic Pseudomonas in 0.1 per cent nitrate agar. First test. Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T, trace, − absent, ? doubtful.*

	MEDIUM F.			MEDIUM BM.		MEDIUM DL.				MEDIUM S								MEDIUM DA.	
	Nitrite present in			Nitrite present in		Nitrate present in				First day		Second day		Fourth day		Seventh day		Nitrite present in	
	1 day	4 days	7 days	4 days	7 days	1 day	2 days	3 days	7 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	4 days	7 days
Culture A	++	+++		−		+	+	++		+	+	++	++	++	++			+	
Culture B	T	++	++	−		+	+	++		+	+	+	++	+	++			+	
Culture C_1	++	+++	+	−		+	+	+		+	+	++	++	++	++			+	
Culture C_2	+	+++	++	−		+	+	++		+	+	+	++	+	++			+	
Culture D_1	+	+++	+	T		T	+	+		+	+	+	++	+	++			+	
Culture D_2	+	++	+	−		T	T	+		+	+	+	++	+	++			+	
Culture D_3	+	++	++	−		+	T	+		+	+	+	++	+	++			+	
Culture X_1	−	+++	+++		−	−	−	−	−			−	+	−	+	−	++		
Culture X_2	−	+++	+++		+	−	−	−	+			T	+	T	+	+	++	−	−
Culture X_3	−	+++	+++		−	−	−	−	−			−	+	T	+	−	++		
Culture a_1	−	−	T		−		−	−	−			−	T	−	T	T	+		
Culture a_2	−	−	T		−		−		−			−	T	−	+	T	+		
Culture a_3	−	T	T		−		−	−	−			−	T	−	++	T	+		
Culture b	−	−	T		−		−	−	−			++	T	+++	++			T	+
Culture c	−	−	T		−		−	−	−			−	T	−	+	T	+		

* In this test the five media were inoculated separately on different days.

These fifteen cultures were tested upon synthetic nitrate media (tests 2–5, table 6), the same media used for the fluorescent organisms. Medium DM (test 2) gave rather surprising results, as nitrite was absent in all but two cases, and in one of these two cases was present in mere traces. This was not due to poor growth in all cases, for very good growth was obtained with cultures A and B and with all substrains of C and D; whereas culture X_2 grew poorly but gave the nitrite test. A later repetition of this test (see table 7) with A, B and all substrains of X showed nitrite to be produced by cultures A and B, but that the nitrite reaction with culture B disappeared after the first day. This suggests that the meaning of the negative reaction in test 2 (table 6) may have been in many cases that the cultures were examined after the nitrite had disappeared.

The poor growth of strains X, a, b, and c, noticed on this medium, was observed on all the other synthetic agars used. This was undoubtedly due to the fact that these media contained no added organic matter except the sugars; for these four strains were found to be unable to attack any sugar.

The cultures were then tested on medium DL (test 3, table 6). A distinct nitrite reaction was obtained promptly with cultures A, B, C_1, C_2, D_1, D_2, and D_3 and on the seventh day with the non-chromogenic strain X_2. No trace of nitrite was observed with any of the cultures which had not produced nitrite in the earlier tests. The correlation between vigor of growth and strength of the nitrite reaction was quite marked. Test 4 was made upon medium S, containing sucrose instead of glucose and lactose, sucrose being used because of its freedom from ammoniacal impurities. Tests for both nitrite and ammonia were made. The results were very striking. Nitrite (at least in traces) was observed with every culture except one of the substrains (X_1) of the non-chromogenic strain. Appreciable amounts of ammonia were observed with every culture, showing nitrate-reduction to have occurred in all cases. As in the previous test, vigor of growth was distinctly correlated with the strength of the nitrite reaction, growth in this case tending to be rather better than on the lactose-glucose medium.

TABLE 7

Nitrate reduction by the orange chromogenic Pseudomonas in 0.1 per cent nitrate agar. Second test. Cultures inoculated in triplicate, but only one tube tested each day. Strength of reaction indicated as follows: +++ very strong, ++ strong, + distinct, T trace, − absent; ? doubtful.*

	MEDIUM P.				MEDIUM DM.				MEDIUM D.				MEDIUM DL.				MEDIUM S								MEDIUM DA.			
	Nitrite present in				Nitrite present in				Nitrite present in				Nitrite present in				First day		Second day		Fifth day		Seventh day		Nitrite present in			
	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	1 day	2 days	5 days	7 days	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	1 day	2 days	5 days	7 days
Culture A...	+++		+++		+	+	++		+	−	+		++	+	+		+		+	+	+	+			+	++	+	
Culture B...	T		+++		+	−	−		++	T	++		T	+	T		++		+	+	+	++			−		+	
Culture E...	+++	+++	+++		+++	+++	+		+	T	+		++	++	+		++		++	T	+	+			++	+	+	
Culture F...	+++	+++	+++		+++	+++	++		++	+	++		T		?		+		++	+	+	+			++	++	++	
Culture G...	−			+++	+		−	−	T		T	+	T		−	+	+				−	++	+	++	++		+	+
Culture X_1..	−			−	−		−	−	−		−	−	−		−	−	−				−	−	−	−	−		−	−
Culture X_2..	−			−	−		−	−	−			−	−		−	−	−				−	T	−	++	−		−	−
Culture X_3..	−			−	−		−	−	−		−	−	−		−	−	−				−	−	−	T	−		−	−
Culture X_4..	−			−	−		−	−	−		−	−	−		−	−												

*In this test the six media were inoculated simultaneously.

Later (test 5, table 6) a few of the cultures were tested upon agar (medium DA) containing ammonium chloride as well as nitrate and glucose. Nitrite was found with all seven of the strains that showed nitrite on nitrate-peptone agar (medium P), also with culture b, the one strain tested that had not shown nitrite on medium P, but not with the one substrain (X_2) of the non-chromogenic organism that was tested.

A few months later those cultures that were still alive were tested again (table 7), together with a few other strains of the same organism. This is a very difficult organism to keep living under laboratory conditions, and only cultures A, B, X_1, X_2, and X_3 were still alive. The strains which had failed to produce nitrite on the medium P had all died. The new cultures studied were:

E, F, G } Three recent isolations from soil, all giving a strong nitrite reaction on Medium P when first isolated.

X_4 A fourth substrain of the non-chromogenic strain, obtained by inoculating X_3 into sterile soil and reisolating after a few weeks.

These nine cultures were inoculated into the same media used in the earlier test and also into medium D.

The only essential difference noted from the results of the earlier tests was that the non-chromogenic cultures had now lost their power of producing nitrite upon any of the media tested, even including medium P. Cultures A, X_1, X_2, and X_4 still gave distinct ammonia reactions in medium S, and X_3 showed a trace of ammonia.

The conclusion drawn from these tests is that this pseudomonad is neither like the colon organisms nor like the fluorescent bacteria in respect to the nitrate-reduction test. Some available organic matter must be present in order to allow good growth; and yet it is possible for some strains to produce good growth without giving the nitrite test. There is evidence that in some cases, at least, this is because the nitrite is converted into ammonia as fast as produced, so that there is no accumulation of nitrite.

CONCLUSIONS

As a result of this work the conclusion was drawn that the nitrate-reduction test, as made by testing for nitrite in "standard" nitrate broth after a definite period of incubation, is not as simple as generally supposed. This test is open to several sources of error:

1. Poor growth. Any organism must be tested in some medium in which it makes good growth. If it grows poorly, the results are likely to be variable (as with the colon organisms in media containing less than 0.2 per cent peptone), and under such conditions absence of nitrite is of no significance. It is probably impossible to find any one medium in which all bacteria make satisfactory growth.

2. Presence of more readily available nitrogen. Some of the cultures studied (fluorescent pseudomonads) seem to be able to reduce nitrate only in the absence of ammoniacal (or amide) nitrogen. Their ability to reduce it does not show, therefore, on ordinary peptone media. Such behavior may sometimes be of diagnostic importance; but the fluorescent cultures studied which showed this characteristic differed in no other observed respect from typical *Ps. fluorescens* (vigorous nitrate-reducer) and are not thought to belong to a separate species.

3. Reduction without accumulation of nitrite. Some organisms (like certain strains of "*B. caudatus*") utilize the nitrite as fast or almost as fast as produced. It may thus be assimilated, converted into ammonia, or converted into free nitrogen. Free nitrogen can generally be detected by gas bubbles in the liquid or cracks in the agar. Ammonia can be detected only if the organism is growing in an ammonia-free medium containing no source of ammonia other than the nitrite; but many organisms are unable to grow under such conditions. Assimilation of the nitrite (either as nitrite or as ammonia) cannot be detected by any simple test.

In case of each of the four species (or groups of species) studied, a different explanation was found necessary to account for cultures showing no nitrite. Only in one case, that of *B. cereus*, did

investigation show the possibility of two species being concerned, one differing from the other in its ability to reduce nitrate. Inasmuch, therefore, as nitrate-reduction on any medium or under any condition whatsoever indicates an organism as a nitrate-reducer, the general conclusion of the present work is that no organism can be safely called a non-nitrate-reducer except as the result of exhaustive tests, too time-consuming to be made in routine bacteriological investigations.

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WATER SUPPLY FOR A SYRINGE BATH

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It is a matter of common laboratory knowledge that nickel plated syringes, steel needles, etc., should be boiled in an alkaline solution. Na_2CO_3 is often employed, although I prefer borax. Some workers have found it desirable to keep syringes in such solutions, in which case they are always ready for use. If tap water is employed, a deposit of lime salts may be deposited at the junction points of the syringes or in the needles, causing troubles of various kinds.

Distilled water will avert such difficulty, but it is necessary to conserve it, and if syringes are used often, it is desirable for obvious reasons, to have just enough water to cover them. I have devised a simple mechanism to supply such a bath with distilled water, almost automatically.

The bottle *A*, figure 1, rests on a shelf in a hood about 2 feet above the level of the bath *F*, which is (viewed from the inside in the figure). The outlet *b*, has a rubber tube connecting with a metal stop-cock inlet *c*, located in the side of the bath as near its bottom as possible. Another stop-cock the inner opening of which is located at *d*, indicates the desired level for the water. The bottle *A* has a two-holed rubber stopper with a piece of glass tubing in either hole. One leads to *d*, the other to the outside air by means of rubber tubing provided with a spring clamp *k*. It is obvious that water will enter the bath until the level rises above *d*, when the resultant negative pressure above the water in the bottle will stop its outflow at *b*. The bath is not perfectly automatic, because the difference in pressure in the bottle and the bath holds some water in *d*, which is released by momentarily

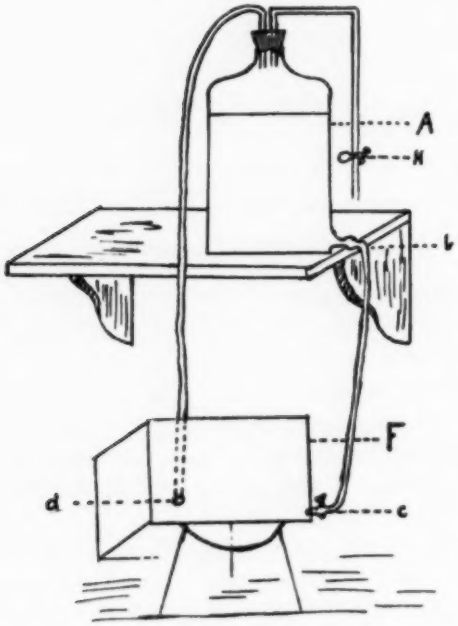

FIG. 1

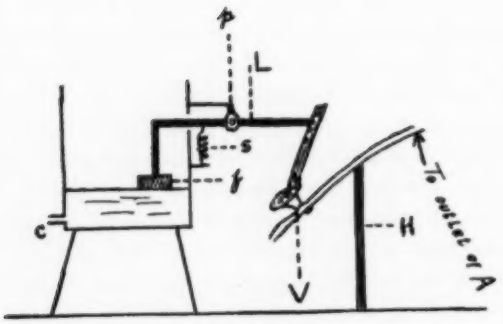

FIG. 2.

opening spring clamp *k*. The pressure is thus equalized, and the level of the water in the bath restored.

If the conditions demanded, it would be a simple matter to devise a strictly automatic mechanism.

As shown in figure 2 one might locate in one corner of the bath a wooden float *f*, connected with a lever *L*, working on a pivot *p*, on the outside surface of the bath's wall. With one end of the lever working against a light coil spring *S*, and the other regulating a valve *V*, controlling the air pressure over the water in *A* (fig. 1), the rise of the water in the bath would indirectly cut off the water supply, which would start to flow again when the evaporation of the water reversed the action of this mechanism. By making the length of the air valve lever adjustable, the level of the water in the bath could be changed at will. In such event stop-cock *d* of the bath as well as the opening in the bottle controlled by clamp *k* could be dispensed with. *H* represents a support for air tube leading to bottle *A*, figure 1.

A MODIFICATION OF THE WRIGHT-BUCHNER ANAEROBIC TUBE

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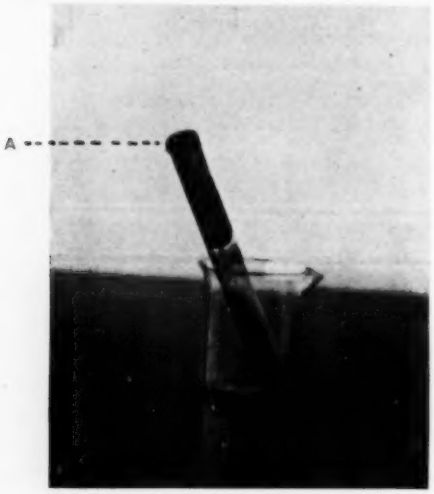
One of the early, anaerobic methods was that of Buchner which involved enclosing the culture tube in an outer airtight test-tube containing pyrogallic acid, employed of course as a reducing agent. Later Wright carried on the entire process in a single tube by depressing the cotton plug and filling the space above it with the acid, after which the tube was corked with a rubber stopper. The tendency for the pyrogallic acid to contaminate the slant and diffuse throughout a non-protein medium has been thought to detract from the value of the method.

With the object of still further simplifying the procedure and making it easier to handle, I have made the following alterations for my own work. I have found that the brand of cotton often employed in bacteriological work is unsuitable for the procedure owing to its permeability. What is known as the "Pearl" brand[1] has yielded most satisfactory results. The plug should be rolled so tightly that it can be tossed about if desired without losing its shape. The loose way in which many plugs are made adds to their permeability, and by no means favors their use in this method.

When the properly fashioned plug is inserted to within a short distance of the top of the slant, a file or some other instrument is run into its center to the depth of a few millimeters. I employ a 5-inch, 3-cornered file with a moderately sharp point about 1 cm. of which is bent at an obtuse angle. With the file in position an ordinary tallow candle is lighted, and paraffin is dropped on top of the plug to a depth of 2 or 3 mm. It should form a

[1] Sold in Ann Arbor, Michigan, by Mack and Company.

layer on top of the plug. When the paraffin has set, pyrogallic crystals are lightly tamped into most of the remaining space. Warm 5 per cent NaOH is pipetted or poured over the crystals, and a soft rubber cap[2] is quickly slipped around the top of the tube, which is preferably a flanged one. A few drops of paraffin from the candle is then let fall on the junction of the tube and cap. With the slanting surface down, the tube is then inclined at a slight angle in the incubator. The inclination is adopted as an additional precaution, but is by no means necessary, for broth cultures can be grown in an upright position with ease



when using the same technique. Following removing of the cap and the pyrogallic residuum, one may open the tube by placing the file in the opening originally made by it in the center of the plug. The tube is heated gently, and the plug removed by spiral revolutions with the file. It is of advantage to discard this plug and use a fresh one to absorb the small amount of pyrogallic that may be present on the walls of the tube. This is usually essential with broth cultures.

[2] These caps are obtained from Parke, Davis and Company, Detroit, and are the same as used by them to cap their stock media.

The whole procedure need occupy but a few minutes. I have used the modification satisfactorily for the past five years. It is neat, clean and reliable. The paraffin coating over the cotton plug prevents the acid from immediately seeking the sides of the tube. It enters the plug slowly at one point only—the center—and when it ultimately permeates the remainder, the supply should be practically exhausted. Thus the tendency to drain down the walls of the tube is almost nil.

This tendency is further diminished by using an elastic rubber cap for a seal rather than a rubber stopper; as the latter, by its encroachment on the space of the tube increases somewhat the intratubal pressure. Furthermore this cap acts as a gauge for the success of the technique in the individual tube—anaerobiosis being indicated by the concavity developed on the caps' surface in response to the diminished intratubal pressure (see fig. at *A*). When blood-agar is used as a medium, its change in color is a sufficient index of successful reduction.

MODIFICATIONS IN THE TECHNIC OF DICK'S PLATE METHOD FOR ISOLATING ANAEROBES

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George F. Dick describes in a recent number of the Journal of Infectious Diseases, (**23,** 577) a very clever and simple method of growing and isolating anaerobes by a plate method. Briefly his method is as follows: Using ordinary petri dishes, the plates for isolating anaerobes are prepared and poured as with ordinary aerobic cultivations; they are then allowed to harden, and a second tube of the same medium is cooled to about 45°, and poured upon the hardened medium. While this latter layer is hardening, paraffin is heated to the smoking point, allowed to cool to about 45° and spread while warm with a spatula over the surface of the agar plate. For examining and fishing colonies developing on these plates Dick suggests that after the colony has developed, a little square of paraffin may be cut out above the colony, and lifted out.

The method of spreading the paraffin over the plates with a knife or spatula is unsatisfactory because it is too slow and is difficult to perform as the paraffin hardens quickly. When it is remembered that almost all strict anaerobes are spore formers there is no reason why the temperature of the paraffin should not be as high as 60° so that it may spread over the solid agar of its own liquidity; but the paraffin at 60° is cooled almost instantly by the cold agar, so there is but slight danger if any even to non-spore formers.

One of the first difficulties encountered after the plates had developed a day or so was the difficulty of seeing the colonies against the white background of the paraffin. Of course transmitted light proved to have advantages in locating the colonies

but it seemed that if a comparatively dark background could be furnished the colonies would be much more clearly visible. After trying numerous dyes, I found but one, Sudan III, a fat soluble red dye, which would dissolve in the liquefied paraffin without trouble, and produce a deep enough color to show up the colonies well. Scharlach R has not been tried but will probably work similarly. Chr. Hansen's butter color dissolves readily in the hot paraffin but the color, a deep orange, is not favorable for detecting colonies, and crystals of some sort are formed in the paraffin when it cools, which also is disadvantageous. Neither of the dyes used diffuses into the medium so that they have no antiseptic or other apparent effect on the cultures. A fat soluble dye of a deep blue, green or black color would be ideal for this use. A large amount of finely powdered charcoal added to the paraffin makes a fairly acceptable background.

Instead of cutting little squares out of the paraffin over the colony it is desired to fish, it has been found much more simple and less destructive to the colony to warm the entire edge of the petri dish slightly in the flame, run a sterile scalpel around the edge and remove the paraffin cover entirely. In this way the whole plate can be examined at leisure and if it is desired to keep this plate culture for future examination, the addition of paraffin may be repeated. When this modification of the original technic is used the addition of dye to the paraffin becomes less necessary.

The paraffin can be used over and over again, and if it becomes dirty from admixtures of colony growths it can be filtered while smoking hot. The paraffin used on the surface of the petri dishes should be perfectly clear as transmitted light as well as reflected light is often necessary for the examination of colonies.

The above modifications in technic have been used successfully in isolating *B. botulinus* from mixed cultures.